RHEOLOGICAL PROPERTIES OF GELATIN, CARRAGEENAN AND LOCUST BEAN GUM MIXTURES

by

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ACKNOWLEDGMENTS

This thesis represents the culmination of an immense period of my life. The man who stood the other side was very different from the one who writes these words of gratitude. I sit here a married man, in my marital home, with a career, a hope and a future! I give thanks for this to my heavenly Father who led me through confusion and anxiety to confidence and joy.

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Lastly, I wish to thank my parents John and Anne Jones who have stood by me loyally and with much love. My final thank you goes to Alexandra who has become my companion and helped open a new chapter of my life and with whom I now face an uncertain, but hopeful future.

Abstract

This thesis reports data on blends of carrageenan (0.3%w/w) and locust bean gum (0.3%w/w) in the presence of biopolymers, particularly gelatin of varying concentration. Particular attention is given to their behaviour on autoclaving since this is relevant to one of the most important applications of these materials as gelling agents in canned meat products.

It was shown there is such 3% gelatin could be found in the gelling system as a result of from collagen in the meat. Gelatin at this level generally reduced the strength of non-autoclaved carrageenan and LBG gels but enhanced autoclaved gels.

Studies of turbidity and rheology suggested that the effect was due to phase separation. Investigation of viscosity after autoclaving produced evidence to support the hypothesis that gelatin protected LBG from thermal degradation. For industrial LBG this was not, however, observed by direct molecular weight measurement, although such effects were seen for pure LBG. Interesting differences between the gel strength response between low and high ionic strength buffers were found.

It was concluded that textural performance in real products was sensitive to a range of factors (salt, impurities, presence of gelatin) and would be interpreted by a combination of degradation and phase separation theory.

Implications for the industry are discussed.

1. Introduction

Several types of canned meat products require a gel-like texture. The textural components can come from the meat in the form of degraded collagen (gelatin) or by the addition of polysaccharides. The gelling matrix discussed in this thesis utilises gelatin, locust bean gum and carrageenan.

1.1. Background and relevance of study

The textural properties of mixtures of biopolymers in petfood gels are of importance commercially from the perspective of assuring product quality. Petfoods are a mixture of materials from both plant and animal origin. They are often subject to change due to the realities of sourcing materials in a competitive market and also where agricultural circumstances limit supply. Historically this has been the case with locust bean gum. These changes compound the already complicated situation of predicting the behaviour of thermodynamically incompatible biopolymers that have been subjected to a severe thermal input. The thesis has sought to elucidate how mixtures of gelatin, carrageenan and locust bean gum behave rheologically and how that affects final texture. Also, what changes occur to these mixtures on heating and how does that affect the texture?

This thesis consists of six chapters. Chapter 1 is a general review of the aforementioned biopolymers used in this study, their structure and function, mechanisms of biopolymer degradation and phase separation in biopolymers. Chapter 2 details the materials and methods used in the research. Chapter 3 details the investigation into the composition of the petfood systems used as the basis of the research conducted in Chapters 4 and 5. Chapter 4 is dedicated to explaining how these biopolymers behave in non-autoclaved conditions, whereas Chapter 5 details the investigation of the effect of severe heating on the degradation behaviour of the biopolymer mixtures. Chapter 6 provides the final discussion and conclusion.

1.2. Biopolymers in pet foods

This section is concerned with the types and nature of biopolymers used in wet-pet food manufacture. Gelatin is the naturally occurring biopolymer, but the severe heating removes much of its gelling functionality. This functionality is replaced by the use of the gelling polysaccharides carrageenan and locust bean gum. These serve to bind water and suspend the meaty material in a gelled matrix. The use of this blend of polysaccharides is to mimic the texture of a gelatin gel. Carrageenan alone gives brittle gels that exude water "syneresis", its combination with locust bean gum makes the gels more compliant and reduces the water loss.

1.2.1. Gelatin

Collagens contain a Gly-X-Y repeating structure (X is often proline and Y is sometimes hydroxyproline). This triplet of amino acids allows collagen chains to twist into a helical structure. Each collagen molecule contains 3 chains twisted around each other to form a triple helix, see figure 1-1. The triple helix gives collagen a rigid structure. It maintains the mechanical integrity of tissues. Gelatin molecular weight distributions are non-monodisperse. The main unit is the α chain. This has a molecular weight around 95kDa. This unit can be associated into β chains (two covalently bound α chains), γ chains (3 α chains), X chains (4 α chains), 1-4 chains (5 to 8 α chains) or Q chains (up to two hundred α chains). There are also many derived structures where amino acids have been lost, these are called A chains with a molecular weight of about 86kDa (Johnston-Banks 1990).

These various molecular weight groups have different effects on the properties of the gelatin. The Q chains are believed to affect viscosity, setting time and setting temperature, whereas, α and β chains govern bloom strength (Johnston-Banks 1990).

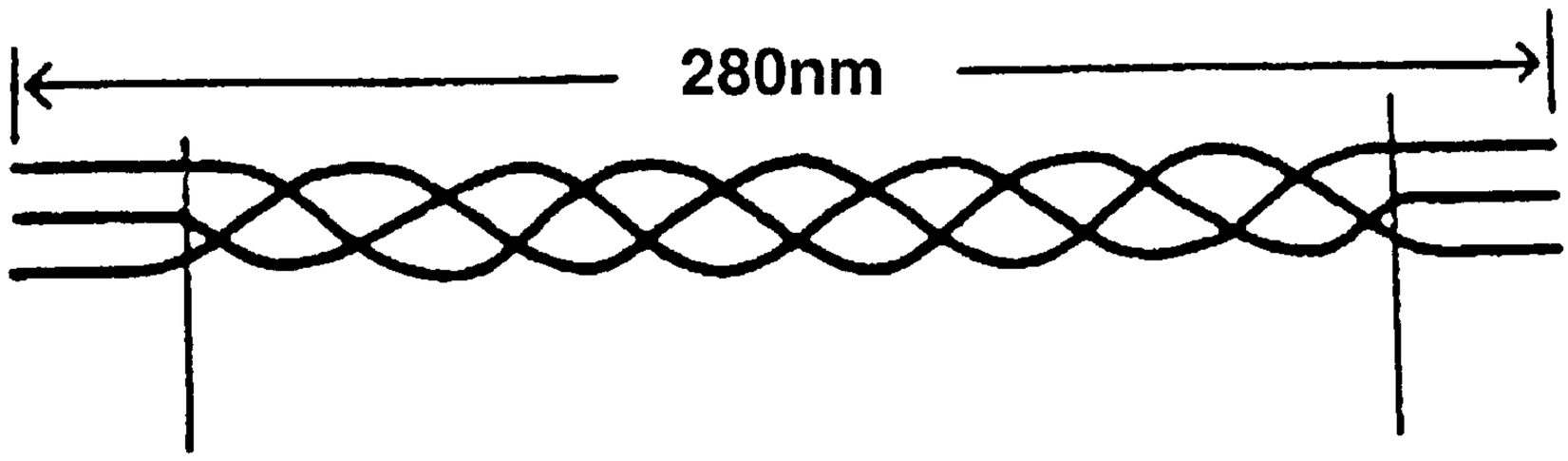

Figure 1-1 Collagen triple helix

A particularly important feature within collagen is the formation of intramolecular (between α chains in the same molecule) and intermolecular cross-links. These cross-links are co-valent (i.e. permanent) and are essential for the high mechanical strength of collagen (Lawrie 1991).

Gelatin is a term for collagen that has been degraded either by heating or chemical treatment. About thirty percent of the protein in humans consists of collagen (Ledward 1986). This is mostly found in skin, bone and connective tissue. Commercial gelatin is processed chemically by the use of acid or alkali (lime) to aid extraction. The method used affects the isoelectric point of the final gelatin. An acid pigskin gelatin has an isoelectric point of pH 9, similar to native collagen, compared to a limed cattle hide gelatin with an isoelectric point of pH 5. The reduction in isoelectric point in limed gelatins is due to the hydrolysis of glutamine and asparagine groups (Johnston-Banks 1990). Acid treatment is used for immature collagens from young animals, whereas lime treatment is used for older animals.

The cooking of animal materials is perhaps the most common method of generating gelatin and certainly the most ancient. Conversion of collagen fibres to soluble gelatin occurs at temperatures above 80°C. Greater cross-linking occurs with increasing age and this increases the resistance of connective tissue to thermal breakdown (Lawrie 1991). Severe thermal processing at autoclave temperature will cause complete conversion of collagen to soluble gelatin regardless of meat type or age of animal.

1.2.1.1. Primary structure and amino acid composition

The amino acid composition is derived from and is very similar to the parent collagen. Collagen is a unique protein because of its large glycine and proline content. It is the only protein to contain large amounts of hydroxyproline, its significant hydroxylysine content is also almost unique. Of the sulfur content, cysteine is notably absent and methionine represents the only sulfur-containing amino acid present. The unique amino acid composition dictates a unique structure. Intramolecular hydrogen bonding is absent due to the high imino acids, which have no hydrogen atom on the peptide bond. Also, di-sulfide bridges are not available to create a tertiary structure (Veis 1964). As a proportion of the total glycine (Gly) accounts for 33% of the protein, proline (Pro) and hydroxyproline (Hyp) 22%, thus the molecular repeating unit can be characterised by (Gly-x-y) where x and y will contain a high proportion of the imino acids (Johnston-Banks 1990).

1.2.2. Locust bean gum

The locust bean (*Ceratonia siliqua*) is a native of the Middle East and is referred to in Christ's parable of the Prodigal Son (Luke 15v16). The gum is obtained by removing the husk from the seeds by roasting then grinding the endosperm. The standard gum often contains testa, which detracts from the quality and produces a milky appearance in solution due to the presence of fats and proteins (Fox 1992).

The generic galactomannan structure comprises a β -(1→4)-linked D-mannose backbone to which single unit α-D-galactosyl residues are attached at O-6, see figure 1-2. The ratio of mannose-galactose depends on the source; the value of 3.5 is typical for locust bean gum (LBG). This ratio dictates the behaviour of LBG in solution as well as its interaction with other biopolymers. The distribution of galactose residues has been found to be irregular, (McCleary 1986), leaving regions of unsubstituted mannose. These regions are believed to enable interactions with other polysaccharides, such as the synergistic interaction with κ-carrageenan (Sand 1982) and also may provide protection from thermal degradation via self-association as

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opposed to guar gum, which lacks unsubstituted mannose regions (McCleary, Clark et al. 1985), (Kok 1999).

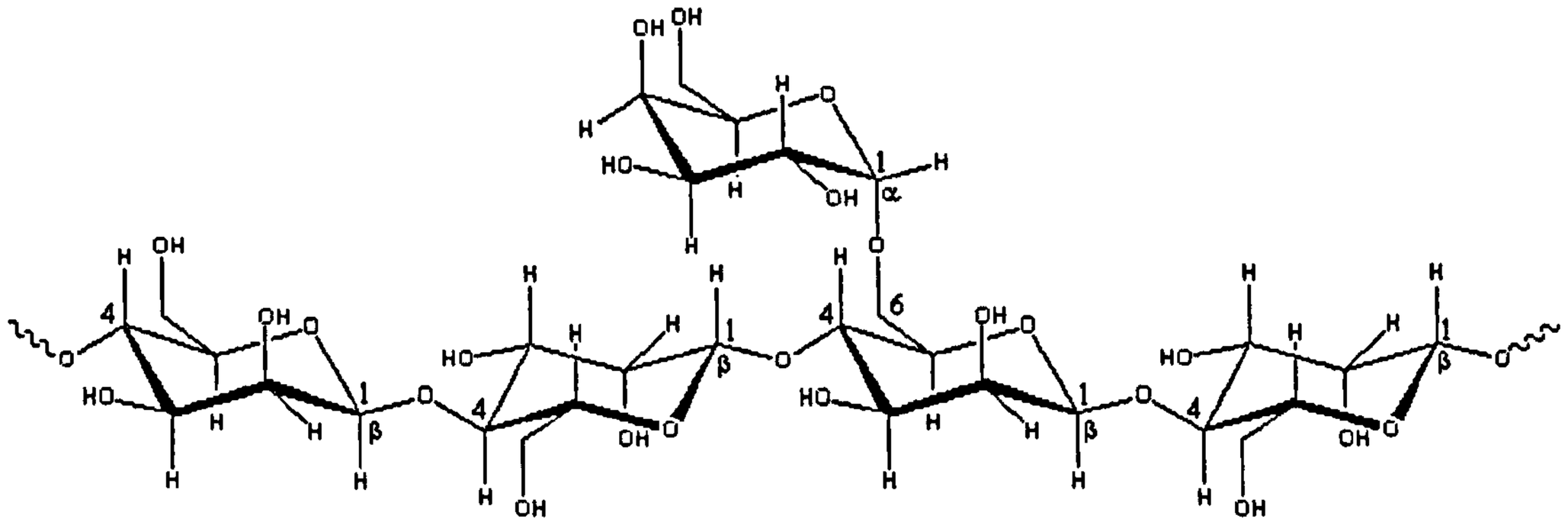

Figure 1-2 Structure of locust bean gum.

LBG is only part soluble in cold water and must be heated to achieve maximum viscosity. Molecular weights from 319 to 1,380kDa have been reported (Gaisford, E. et al. 1986), (Doublier and Launay 1981).

1.2.3. *K*-carrageenan

Carrageenans are galactan polysaccharides that occur in red seaweed (*Rhodophyceae*). Carrageenans are linear sulfated polysaccharides made up of repeating galactose disaccharides called carrabiose. The carrabiose units comprise alternately linked α(1,3) and β(1,4) D galactopyranose. In κ-carrageenan the sulfation on the β(1,4) galactopyranose-4-sulfate results in 1 negative charge per carrabiose unit, see figure 1-3, (Stanley 1990).

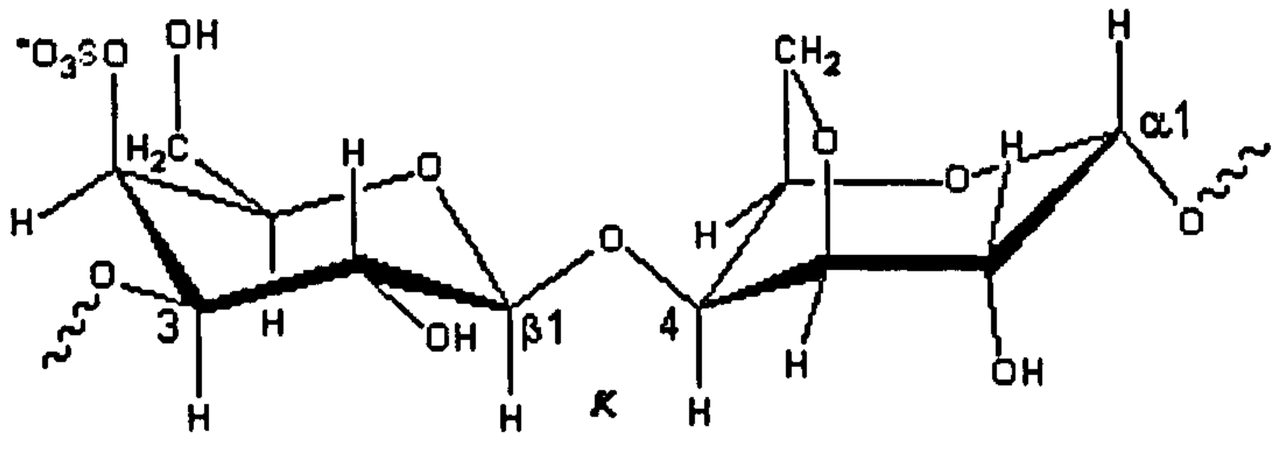

Figure 1-3 Representation of κ-carrageenan (-(1→3)-β-D-galactopyranose-4-sulfate-(1→4)-3,6-anhydro-α-D-galactopyranose-(1→3)-).

The gelation properties of κ-carrageenan will be discussed later, but the presence of sulfate on the galactose backbone and makes possible interactions with cations, in particular potassium. The interaction with cations results in typical dissolution temperature exceeding 70°C.

Carrageenan is obtained via a purification process that seeks to liberate the biopolymer from the originating seaweed. This is done principally by heating in potassium hydroxide, a consequence of this is to remove sulfate and it is believed that κ-carrageenan is derived from a pre-cursor, μ-carrageenan, which contains an extra sulfate. Figure 1-4 shows the process of obtaining semi-refined carrageenan, as is used in industrial applications.

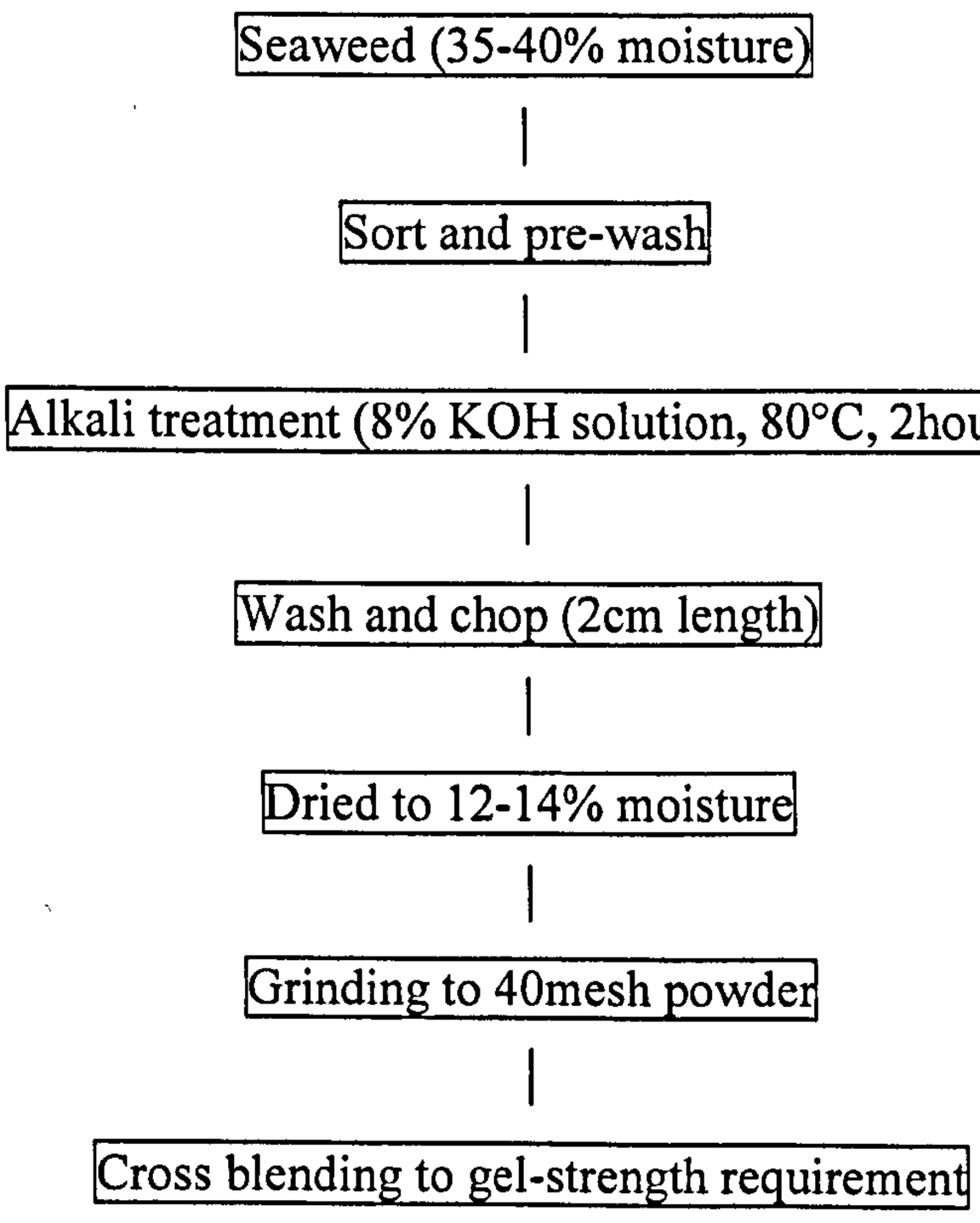

Figure 1-4 Semi-refined carrageenan process.

1.3. Rheological behaviour of biopolymers in petfoods

The macromolecules described are used to give the textural attributes required by the product. Some of the terminology used when describing the rheological properties of the systems is given below.

Rheology is the study of the flow and deformation behaviour of materials. All materials lie on a continuum between the ideal or Newtonian fluid and the ideal or Hookean solid. Newtonian fluids have a viscosity that is independent of the strain rate applied. Viscosity is defined as:

$$\eta = \frac{\sigma}{\dot{\gamma}} \qquad \text{Equation 1-1}$$

Where η is the viscosity (Pa.s), σ is the shear stress (Pa) and $\dot{\gamma}$ is the shear rate (s^{-1}). Viscosity is affected by other physical factors of which the most important is temperature.

1.3.1. Flow behaviour

Most biopolymers do not have Newtonian flow behaviour but deviate from it in some way. Using the Cross model it is possible to describe these deviations:

$$\eta = \eta_\infty + \frac{\eta_0 - \eta_\infty}{1 + K_1(\dot{\gamma})^{2/3}} \qquad \text{Equation 1-2}$$

Here η_∞ is the infinite shear viscosity, η_0 is the zero-shear viscosity and K is a constant.

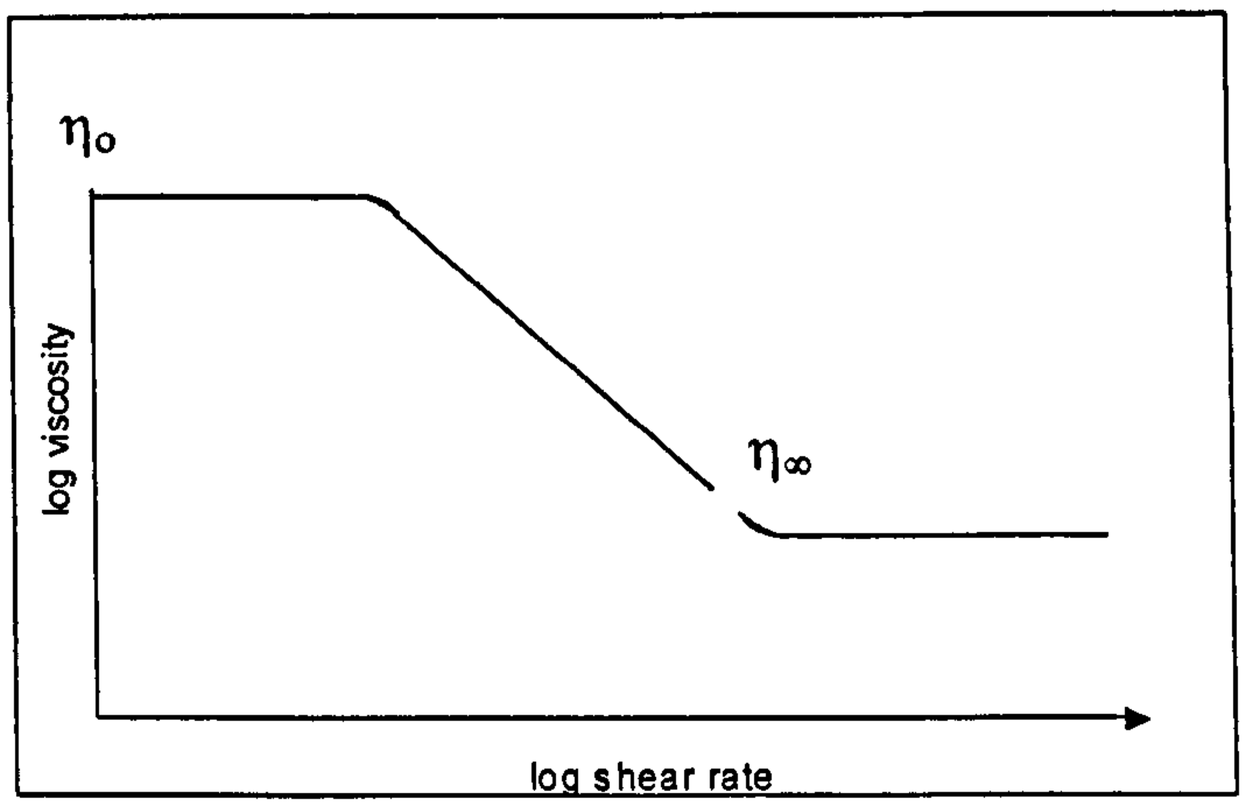

Figure 1-5 Viscosity function of non-crosslinking biopolymers (like galactomannans)

Viscosity is also dependent on the concentration. At low concentrations the zero-shear viscosity (η_0) increases almost linearly with increasing concentration. A slope of ~1.3 has been reported where η_0 is plotted against concentration on a double-logarithmic graph (Morris 1984). As the concentration increases a critical point is reached and the slope increases abruptly to a value typically around 3.3. See figure 1-6.

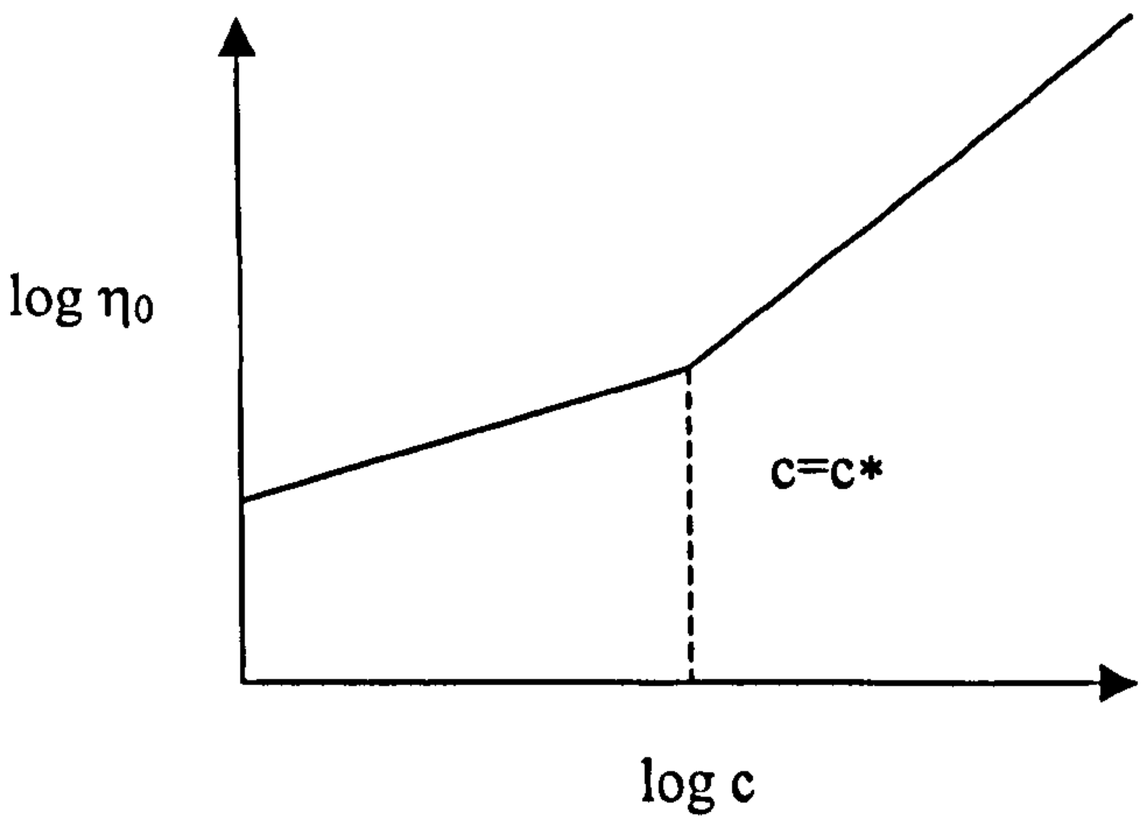

Figure 1-6 The concentration (c) dependence of zero shear viscosity (η_0) for a typical "random coil" hydrocolloid (Morris, 1984).

This abrupt increase in viscosity is believed to be caused by a change from a solution of freely moving biopolymer coils to a region of entangled chains as the concentration exceeds the critical value c*.

Molecular weight is another factor important in determining the viscosity of biopolymer solutions. The intrinsic viscosity [η] is dependent on the shape and size of a biopolymer and provides information about the molecular size and conformation. It also gives information about solvent interactions. Intrinsic viscosity is obtained by measuring the viscosity of progressively more dilute solutions of biopolymer and extrapolating to zero concentration. Intrinsic viscosity is measured in the units of decilitre per gram. Table 1-1 shows reported values for the biopolymers used in this study.

	[η] (dl/g)	Reference
Gelatin	0.69	Courts and Stainsby, 1958 cited by (Harding 1998)
Locust Bean Gum	13.2	(Kok 1999)
K-carrageenan	6.5	(Myslabodski, Stancioff et al. 1996)

Table 1-1 Reported intrinsic viscosities for the biopolymers used in this work.

Molecular weight, M, can be related to intrinsic viscosity by the Mark-Houwink relationship below:

$$[\eta] = K' M^{a}$$ Equation 1-3

The parameters *K'* and *a* depend on the conformation of the biopolymer.

1.3.2. Gelation

The ability of biopolymers to form gelled networks greatly enhances their usefulness at controlling the movement of water and permits the creation of solid-like structures in which food materials can be suspended. For a gel to exist a permanent network must be present, this occurs due to the association of biopolymer chains to form junction zones (Morris 1998). Gels are characterised by viscoelastic rheological behaviour.

Purely viscous fluids dissipate all the energy obtained through deformation; none of this energy is recovered when the deforming stress is removed. The alternative case is where all the energy is retained within the material and completely recoverable on removal of the stress i.e. purely elastic. Viscoelastic materials have properties that are mixtures of these two attributes. Two moduli, G' – the elastic modulus and G" – the viscous are defined thus:

$$G' = \frac{\sigma_0}{\gamma_0}\cos\delta$$ Equation 1-4

$$G'' = \frac{\sigma_0}{\gamma_0}\sin\delta$$ Equation 1-5

Where σ_0 is the shear stress, γ_0 is the amplitude of the strain response and δ is the phase angle. For elastic materials, such as gels, the phase angle will be as low as 1° i.e. the applied and measured stress response will almost be in phase. Viscous materials have phase angles approaching 90° as the measured stress is out of phase with the applied stress.

1.3.2.1. Measurement of gelation

Whilst it is possible to visually assess the structure of many gels, it is necessary to have an objective assessment of when gelation has occurred. In the case of gelatin and carrageenan the point of gelation is temperature dependent. For the work conducted in this thesis gelation is said to have occurred at a phase angle of 45° as described by Winter and Chambon, 1986.

1.3.3. Functionality of biopolymers

1.3.3.1. Gelatin

Gelatin exists in random coil state above 40°C in aqueous solutions configuration (Ross-Murphy 1992). Above the setting temperature the viscosity of solutions is unusually low. Relatively high concentration solutions can be prepared with appropriate handling. Gelatin further enhances its unique properties by the Newtonian behaviour of its solutions, which differs from polysaccharides at similar molecular weights (Wulansari, Mitchell et al. 1998).

Gelatin gels are thermoreversible, and have a melting range of between 27 and 34°C (Poppe 1992), dependent on concentration. Gelation in gelatin involves a coil-helix transition, triple helix formation and aggregation. The transition observed here is very slow compared to other gelling systems, such as carrageenan (Ross-Murphy 1992). Gelatin gels mature with time and existing linkages continually reorganise themselves and progressively more of the peptides find themselves in the ordered state (Ross-Murphy 1992). The junction zones consist of aggregated triple helices, which are thought to be very similar to the native collagen structures.

Gel strength in gelatin is characterised by the Bloom value. The Bloom strength is the weight in grams required to depress a 0.5inch diameter probe 4mm into 6.67% gelatin

gel. The gels are cured at 10°C for 18 hours. Bloom values range from 50-300 (Johnston-Banks 1990).

1.3.3.2. Locust bean gum

LBG has a random coil structure. The movement of these coils is dependent on concentration and as this increases the coils move from between what are called the dilute to the entanglement domains. This transition is accompanied by an increase in viscosity with concentration. LBG produces highly viscous aqueous solutions that display pseudoplastic flow behaviour in the entanglement region. This behaviour is believed to be due to the de-entanglement of the coils as the shear field increases causing a viscosity decrease. This is a reversible process, (Fox 1992).

1.3.3.3. *K*-carrageenan

Below the coil-helix transition temperature, *K*-carrageenan forms double helices, which require the presence of specific cations for gelation to proceed, see figure 1-7. This difference in mechanism creates a wide range of melting/gelation temperatures dependent on cation concentration, and it generates hysteresis. *K*-Carrageenan at 1% will vary in gelation temperature between 15 and 60°C over 0.05 to 0.8% K+ (Thomas 1992), melting temperatures can exceed 90°C.

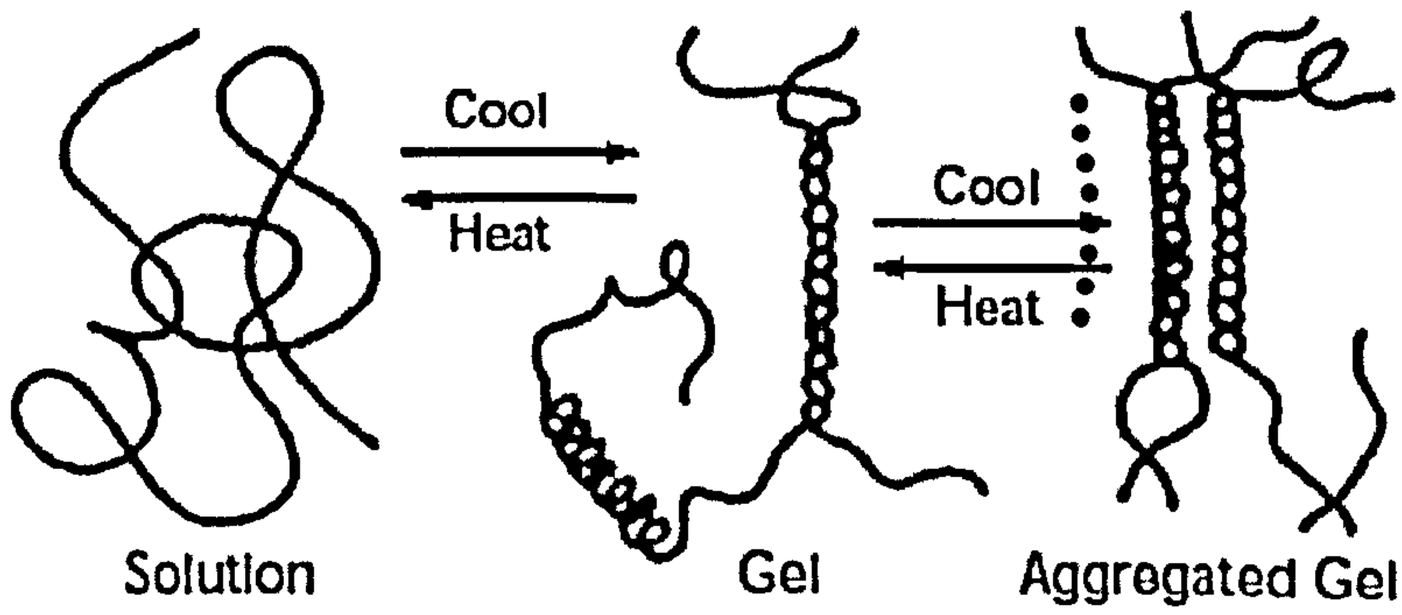

Figure 1-7 Domain model for carrageenan gelation. • - gel promoting cation.

1.3.3.4. Synergism between *K*-carrageenan and locust bean gum

Whilst LBG does not gel under normal conditions it does interact with *K*-carrageenan to produce gels of a lower total biopolymer concentration than that required for *K*-carrageenan gelation alone (Dea and Morrison 1975). A full analysis of the possible mechanisms for this 'synergism' is given by V. J. Morris in "Functional Properties of Food Macromolecules", eds. Mitchell and Ledward, 1998. The traditional view is that unsubstituted regions of the mannan backbone bind to the helical part of the carrageenan.

The three biopolymers described above are to be used for the production of a textured product. However, it is important to recognise that gelatin and the polysaccharides may not be perfectly mixed in all situations. The underlying mechanisms for this demixing are described in the next section.

1.4. Phase separation in biopolymer mixtures

1.4.1. Background theory

Mixing processes are subject to entropic forces, which promote the even mixing of the constituents. Where conditions are placed on a system that tend to lower the entropy, the system becomes thermodynamically unstable. For example, in figure 1-8 below entropy will disperse the particles chaotically, in B, rather than group them as in A:

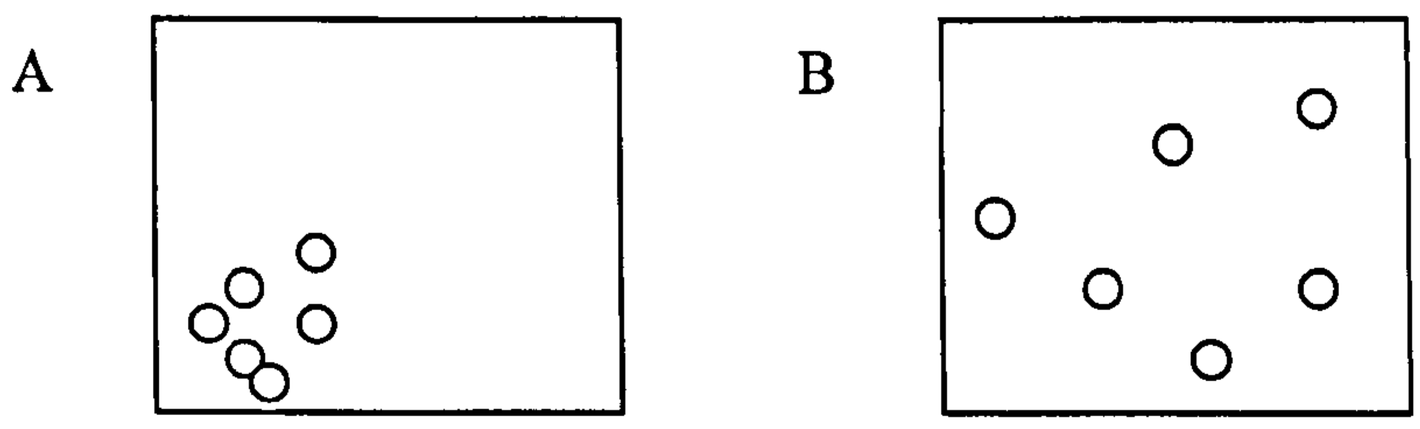

Figure 1-8 Entropy tends to promote even mixing.

Phase separation is often observed in mixtures of liquids and is related to unfavourable interactions, which dominate the entropic forces. The most obvious example is the observable separation of oil in water. Liquid mixtures, such as polymers dissolved in a solvent, can be said to be partially miscible if their mixing is dependent on independent factors such as temperature, composition and proportion of components, (Atkins 1992). It is possible to construct a diagram based on these factors.

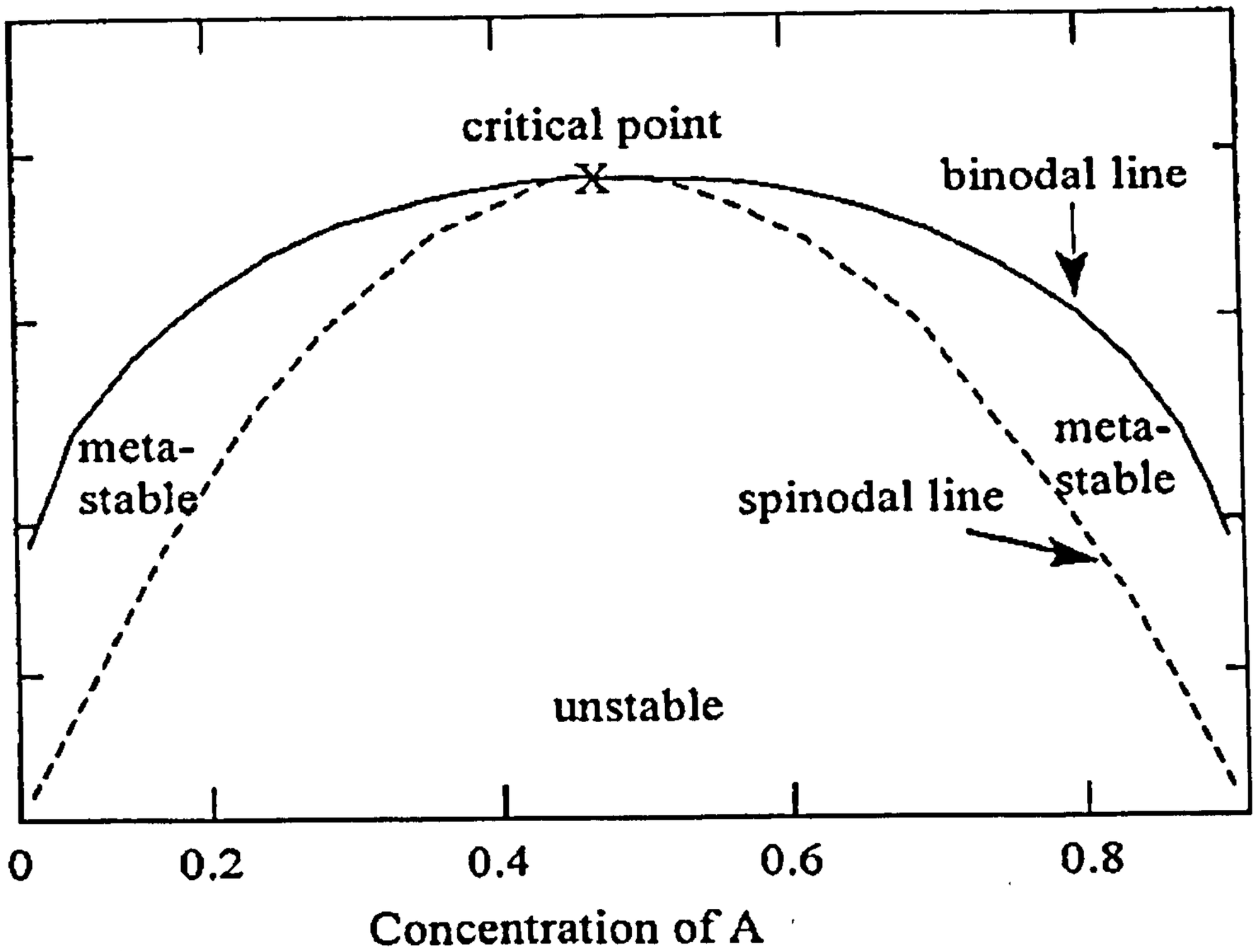

Figure 1-9 Phase diagram of binary mixture, (Donald, Durrani et al. 1995).

Figure 1-9 shows the relationship between temperature and phase separation for a two-component system. At a critical temperature phase separation can occur for any proportion of the binary mixture. The solid line that denotes the point at which phase separation can occur is called the binodal or coexistence curve. The dotted line is termed the spinodal line, which denotes the limit of stability. Below the spinodal in the unstable region phase separation will occur. Between the spiniodal and the binodal exists the meta-stable region where concentration fluctuations prevent phase separation occurring spontaneously. The barrier to phase separation occurring concerns free energy, which the system lowers if possible. When phase separation occurs free energy is gained by the new interfaces created. An overall loss in free energy must occur in the event of phase separation. In the metastable region phase separation can occur due to homogeneous nucleation and growth where the concentration fluctuations cause 'seed' areas, which grow with time. The presence of third party 'seed' particles create nucleation sites and lead to phase separation by heterogeneous nucleation and growth. Both homo and heterogeneous nucleation processes require activation energy. Seed particles lower the activation energy

required (Donald, Durrani et al. 1995), (Clark 1995). If a system is cooled rapidly into the unstable region phase separation can occur due to spinodal decomposition, which requires no activation energy (Clark 1995).

$$\Delta G = \Delta H - T\Delta S \qquad \text{Equation 1-6}$$

Equation 1-6 shows the relationship between free energy (G), enthalpy (H) and entropy (S) for a mixing process. Since systems tend to increase their entropy and thereby reduce their free energy, mixing can only occur when the TΔS exceeds the enthalpy term. The relationship between temperature and mixing can be seen as greater thermal motion promoting miscibility, and results in a critical temperature above which phase separation can not occur (Atkins 1992).

In mixtures of polymers phase separation occurs due to limited miscibility (Piculell, Bergfeldt et al. 1995). What this means in thermodynamic terms is that a phenomenon, which overrides the entropy of mixing, is present. The entropy of mixing has a lesser effect in polymer mixtures due to the relatively few molecules that are present; this allows the interaction energies between polymer chains to determine the behaviour (Morris 1990). These interactions may be neutral, repulsive or attractive. For long polymers weak repulsive forces between two different polymer kinds result in phase separation (Gustafsson, Wennerstrom et al. 1986).

1.4.1.1. Flory-Huggins Lattice Model

The Flory-Huggins theory describing polymer phase separation in organic solvents is widely accepted (Gustafsson, Wennerstrom et al. 1986). It has been shown to be useful in describing phase separation since it is based on polymer interactions as the central concept, which is believed to be the determining factor in phase separation.

For a simple mixture of two molecules the mixture may be envisaged as a lattice, each site of the lattice may be occupied by a segment of the molecule (Flory 1953). Flory defines the entropy of mixing per lattice site as:

$$\Delta S_{mix,\,site} = -k\left[\frac{\phi_1}{r_1 \ln \phi_1} + \frac{\phi_2}{r_2 \ln \phi_2}\right] \qquad \text{Equation 1-7}$$

ϕ - Volume fraction

r_1 - Relative length

k - Boltzmann's constant

Equation 1-7 shows the dependence of the entropy of mixing ΔS on the length of the molecule. As the relative length increases, the entropy becomes very small (Piculell, Bergfeldt et al. 1995). This is due to the reduction of the number of configurations possible on the lattice.

$$\chi_{ij} = \frac{z}{kT}\left(w_{ij} - \left(\frac{w_{ii} + w_{jj}}{2}\right)\right) \qquad \text{Equation 1-8}$$

z - number of nearest neighbours to any lattice site.

W - free energies of interaction between segments of i and j when these occupy neighbouring sites on the lattice.

Equation 1-8 introduces the Flory-Huggins pair interaction parameter for components i and j in a mixture, based on the interaction free energies between the segments of i

and j. Attractive pair interactions are negative, whereas repulsive pair interactions are positive.

Scott gives the critical interaction parameter value, which the pair interaction parameter has to exceed for phase separation to occur (Scott 1949).

$$\chi_{c12} = \frac{\left(\sqrt{r_1} + \sqrt{r_2}\right)^2}{2r_1 r_2} \qquad \text{Equation 1-9}$$

For a monomeric mixture $\chi c_{12} = 2$, for a polymeric mixture χc_{12} tends to zero as r becomes infinitely large. Hence, it can be seen that polymer incompatibility will occur where slight repulsion exists between very long polymers (Piculell, Bergfeldt et al. 1995).

In a ternary system of solvent (1) and two polymers (2 and 3), the Flory-Huggins interaction parameters have the form χ_{12}, χ_{13} and χ_{23} (Dickinson and Stainsby 1982). Where repulsion between the solvent and the polymers exists large values for χ_{12}, and χ_{13} are returned, both polymers separate into one phase. This is the case for a poor solvent. Where χ_{23} becomes large, separation results in the polymers excluding the other from its phase and may be seen as segregative in nature (Piculell and Lindman 1992). Theoretical diagrams show that as the term χ_{23} increases above the critical interaction parameter, the region of phase separation increases (Piculell, Bergfeldt et al. 1995).

1.4.1.2. Assessment of the Flory-Huggins model

The Flory-Huggins theory shows how increasing the molecule chain length allows repulsive interaction energies to cause phase separation, dominating the entropy of mixing. The theory also introduces the concept of the interaction parameter, χ, which gives the relative affinity between the components of a system. Where there are large interaction energies between two polymers, phase separation is more likely to occur.

The theory makes assumptions which need to be highlighted. For example, it assumes complete flexibility of the polymer chain and random distribution of the segments. This may or may not be the case (Dickinson and Stainsby 1982). Also, it assumes that the monomer unit of the polymer occupies the same volume as a solvent molecule (Gustafsson, Wennerstrom et al. 1986). The theory is based on non-ionic polymers in ternary systems, although it is possible to screen ionic effects using excess salt (Piculell, Bergfeldt et al. 1995). Polyelectrolytes in solution show improved miscibility due to the entropic effect of the dissociated counter ions. This effect favours even mixing of the counter ions throughout the system (Piculell, Bergfeldt et al. 1995).

Application of theoretical models, such as Flory-Huggins, to biopolymer mixtures of the nature found in food systems requires consideration. The Flory-Huggins theory is based on non-aqueous systems (Gustafsson, Wennerstrom et al. 1986). Food systems are unlikely to be mixtures of pure components and will contain contaminating material.

1.4.1.3. Classification of phase behaviour in biopolymer mixtures

Piculell, L., Bergfeldt, K. and Nilsson, S. (1995), define three types of phase behaviour observed for a mixture of polymers in solvent:

1. Segragative Phase Separation, the most common form, where two phases are formed, due to polymer incompatability. Each phase is enriched in one of the biopolymers.
2. Associative Phase Separation, where electrostatic attraction creates two phases, one of which is enriched in both biopolymers.
3. Borderline Phase Separation, here, one of the biopolymers is distributed equally between the two phases.

Phase separation in mixtures of two biopolymers plus water as solvent, can be represented by phase diagrams, which show phase behaviour as a function of concentration. Figure 1-10 shows a phase diagram for a protein-polysaccharide mixture. The area above the binodal curve is the phase separated region. The binodal

is often termed the cloud-point curve as it denotes the mixture proportion at which turbidity is observed.

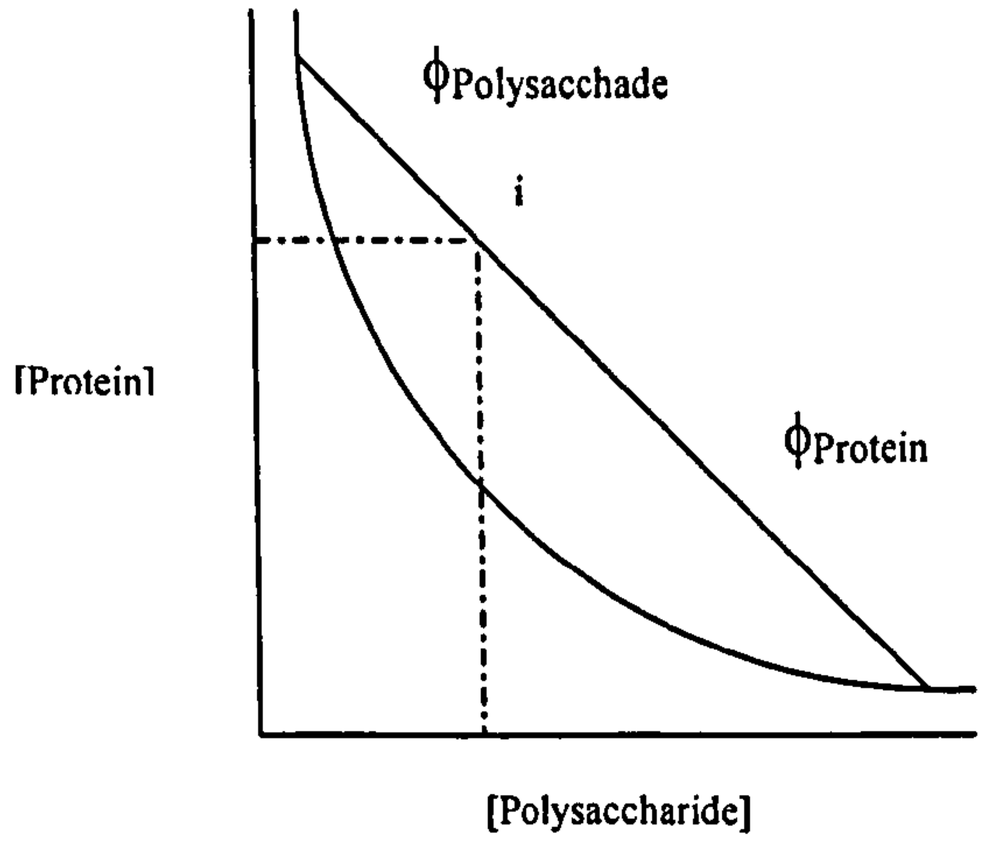

Figure 1-10 A phase diagram

A tie-line can be drawn from any two points on the binodal curve. This is found by measuring the concentration of constituents in each phase. Any mixture that shares the same tie-line will separate into two phases of the same concentration of each polymer. The volume fraction ϕ of each phase will vary.

The volume fraction ϕ in each phase is inversely proportional to the concentration of the dominant polymer in any phase, i.e. the greater the volume the lesser the degree of enrichment in that phase. This is known as the lever rule (Atkins 1992).

1.4.1.4. Methods of measuring phase separation in biopolymer mixtures

In order to construct a phase diagram for a biopolymer mixture it is necessary to first, determine whether phase separation has occurred, second, separate the two phases and third, determine the biopolymer concentration in each phase. Workers have used different methods to construct phase diagrams for systems relevant to the studies described in this thesis. Such work includes that of Michon, Cuvelier et al. 1995 who used visual observation to classify mixtures of iota-carrageenan and a high iso-electric

point gelatin as clear, cloudy, opaque and phase separated. The phase diagrams were constructed using the initial concentrations and were divided into areas based on the classifications. There is the inference that phase separation only occurs when two macroscopic phases are present.

Clewlow, Rowe et al. 1995 centrifuged mixtures of low-methoxy pectin and gelatin mixtures to achieve macroscopic phases. The gelatin concentration was determined using a protein determination method (Bradford 1976), whilst the pectin was determined using a phenol-sulfuric acid method (Dubois, Gilles et al. 1956).

Macro phase separated mixtures of amylopectin and gelatin were analysed using Fourier Transform Infrared spectroscopy by Donald, Durrani et al. 1995. This work also involved the use of time-resolved small angle light scattering to study the kinetics of phase separation.

Phase separation studies in mixtures of proteins and polysaccharides by Hoskins, Robb et al. 1996, used the onset of turbidity at 650nm absorbance as the phase separation point. Macroscopic phase separation appears to have occurred after two hours and the concentrations in each phase was determined using fast permeation liquid chromatography.

Phase separation in gelatin and maltodextrin mixtures was qualified by the onset of cloudiness and the two phases obtained by centrifugation. The layers were weighed and the concentration of biopolymers in each was determined using the Drude method (Kasapis, Morris et al. 1993).

Increase in absorbance beyond 2-3% at A_{490} was taken as the binodal point for k-carrageenan/gelatin mixtures. Concentrations in insoluble complexes were determined by removing the carrageenan from the complex by salt precipitation and absorbance at 240nm to determine the gelatin. The carrageenan was determined by subtraction from the total dry mass (Antonov and Goncalves 1999).

Rheology can also be used to give an indication of phase behaviour. Viscoeleastic measurements coupled with microscopy of gelatin/agar mixtures showed gel

weakening of agar gels with increasing gelatin concentration (Clark, Richardson et al. 1983). Phase inversion was achieved at 2.5% gelatin for a 1% agar gel. The use of viscoselastic measurement has also been used to show salt induced phase inversion in gellan/gelatin gels (Papageorgiou, Kasapis et al. 1994). A melting temperature shift from >95°C to <30°C was observed in 0.5% gellan and 5% gelatin gels between 500 and 750mM NaCl.

1.4.1.5. Factors influencing phase separation in biopolymer mixtures

All phase diagrams based on empirical observation are subject to the actual conditions under which the observations were taken. It is therefore essential to have as much knowledge of the starting materials and the conditions of observation as possible. These factors may be grouped as follows:

1. factors intrinsic to the biopolymer, such as charge and molecular weight
2. solution characteristics, ionic strength and pH
3. temperature

From the Flory-Huggins theory, interaction energies between biopolymers and their length can be seen to be important. For any given biopolymer, chain lengths and distributions vary considerably. Donald, Durrani et al. 1995 show that increasing the molecular weight in amylopectin increases the two-phase area on the phase diagram. Greater biopolymer flexibility increases the number of unfavourable contacts and can promote phase separation. In the case of polysaccharides, where conformational freedom is often restricted for steric reasons, polymer exclusion is reduced (Morris 1990). The presence of charges on the biopolymer greatly increases the entropy of mixing and increases miscibilty, see figure 1-11. This entropy promotes the dissociated counter-ions to distribute evenly through the system (Piculell, Bergfeldt et al. 1995).

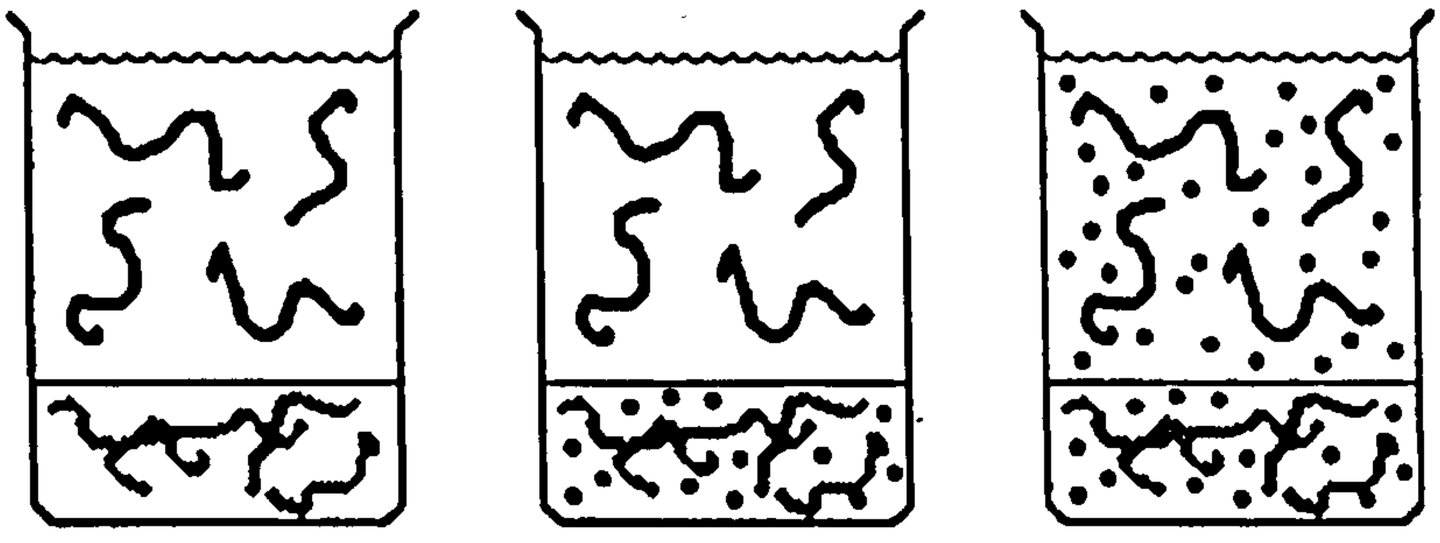

Figure 1-11 Counter-ions promote miscibility, (Piculell, Iliopoulos et al. 1991).

The properties of the aqueous environment are important. The presence of salt can promote miscibility in systems where electrostatic forces promote association, by masking the charge effects. This has been demonstrated by Michon et al 1995 in oppositely charged gelatin and carrageenan. In mixtures of similarly charged polyelectrolytes, increasing the ionic strength of the solution favours phase separation by segragation (Piculell, Iliopoulos et al. 1991).

Whether phase separation will occur for a given system is temperature dependent and results in a critical temperature above which phase separation will not occur. This is due to increased molecular motion at higher temperatures (Atkins 1992).

1.4.2. Phase behaviour in mixed gels

Many systems, such as food, are often non-equlibrium state sytems and may exist as glasses or gels (Donald, Durrani et al. 1995), which impose kinetic limitations on the thermodynamic processes. The eventual state of the system is then a result of the nature and extent of the phase separation process as well as the gelation process. In the case of carrageenan and gelatin mixtures the main factors which dictate the mixture properties are the biopolymer concentration, pH, ionic strength, the temperature and the gelation rate. Out of this complexity four schemes have been presented for the structure of gelling biopolymer mixtures (Cairns, Morris et al. 1986).

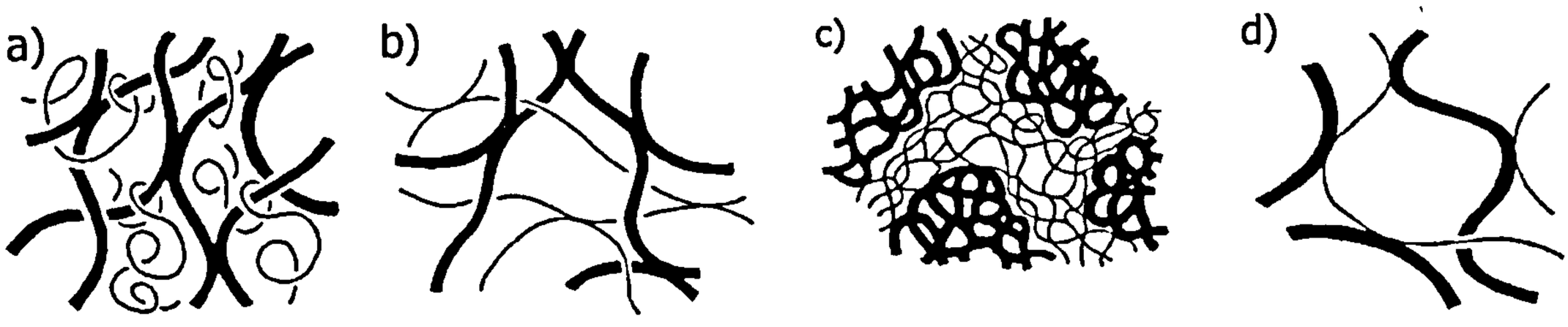

Figure 1-12 Mixed Gel Networks. a) network containing second biopolymer, b) interpenetrating network, c) phase separated network and d) coupled network. (Cairns et al. 1987).

For two biopolymers A and B, the simplest structure occurs when A forms the gel matrix, in which B is uniformly dispersed (Fig. 1-12a). This is likely at high degrees of biopolymer compatibility, such as low concentrations and molecular weight of B. The other schemes describe networks where both biopolymers contribute to the network. Interpenetrating networks consist of two intertwined independent networks (Fig 1-12b). Where phase separation leads to demixing prior to gelation, A and B will form phase separated networks (Fig 1-12c). Lastly, where A binds to B by new heterotypic junction zones (Morris 1998), the gel is a coupled network (Fig 1-12d). The synergistic behaviour of carrageenan and locust bean gum is an example of a coupled network.

Where extensive phase separation leads to formation of discrete inclusions, a dramatic reduction in the mechanical strength of the gelled mixture occurs. This is because the

phase-separated inclusions provide fracture initiation points when the gel experiences stresses. The size of the inclusions is also important. This has been demonstrated in gelatin/agar mixtures, where gelled agar pieces are dispersed in a gelatin disperse phase (Fujii, Kajita et al. 2000). The rupture stress was shown to decrease as a function of increasing inclusion segment length. The movement of water between phases in a gel mixture can also affect the structure. The deswelling of agar networks in the presence of molten gelatin has been demonstrated (Clark, Richardson et al. 1983).

Phase separation between carrageenan and gelatin has been reported in some depth and useful parallels can be drawn from other polysaccharide/gelatin mixtures. In viscoelastic studies (Michon, Cuvelier et al. 1996a) and visual assessment, (Michon, Cuvelier et al. 1996b) the concentration dependence on phase separation between *iota*-carrageenan and type A gelatin was investigated. Careful manipulation of the carrageenan enabled the electrostatic interactions with gelatin to be studied, which were found to occur well above the isoelectric point of the gelatin (Antonov and Goncalves 1999). In studies with the starch derivative, dextran, and gelatin, the concentration required to reduce gel compliance (stress/strain) was found to increase for dextran fractions of decreasing molecular weight (Tolstoguzov 1998).

1.5. Changes to biopolymers during heating

As discussed in the phase separation section, a factor to be considered is the size of the biopolymers. A feature of the manufacture of canned products is the high temperatures that the materials experience causing the biopolymers to undergo changes.

1.5.1. Depolymerisation

Three mechanisms exist to describe the depolymerisation of biopolymers, the hydrolysis of glycosidic or peptide bonds, beta elimination and free radical attack.

1.5.1.1. Hydrolysis

Hydrolysis is defined as the decomposition or alteration of a substance with water. The energy requirement for hydrolysis to occur can be provided for by thermal energy. The degree of depolymerisation is dependent on reaction time, hydrolysis reagent and temperature (Bradley 1989).

1.5.1.2. Beta elimination

Beta-elimination is a term used to describe the formation of a double or triple bond by the loss of groups on adjacent atoms. This process is particularly dependent on pH due to the requirement of hydroxyl ions in the reaction. This requirement means that this type of reaction occurs more rapidly at neutral and alkali pH.

1.5.1.3. Free-radical attack

Free radicals are species having one or more unpaired electrons. These have been shown to cause biopolymer degradation during thermal treatment (Wellington 1983). Viscosity studies have been used to support the idea that radical attack causes depolymerisation through viscosity reduction (Gilbert, King et al. 1984). Free radical reactions are believed to progress through three stages; initiation, propagation and termination.

1.5.2. Maillard reactions

The reaction of reducing sugars with amino functional groups on proteins, such as gelatin, is promoted during thermal processing. This is partly explained by increased rates of reaction, but in our case, the cooking process releases the protein to engage in such reactions. Whilst Maillard crosslinking has been shown to have gelation properties, (Armstrong, Hill et al. 1994), the most significant factor is the reduction in protein isoelectric point caused by reaction of positively charged functional groups, such as lysine, (Mohammed, S.E. et al. 2000).

This concludes the introduction of this thesis. In the following sections the compositional factors of petfood gels are discussed and their impact on final gel texture.

2. Materials and Methods

2.1. Materials

A crude, technical grade, locust bean gum (cLBG) was obtained from a commercial source. Locust bean gum was also obtained from Sigma, UK and Meyhall Chemicals, A. G., Kreuzlingen, Switzerland. This last sample was purified using the ethanol precipitation method of Girhammar and Nair 1992 as cited by Picout, Ross-Murphy et al. 2002.

A predominantly kappa carrageenan sample was obtained from Sigma, UK, other pure types of *iota*, *kappa* and *lambda* carrageenan were obtained from the same supplier. Semi-refined carrageenan was also extracted from the seaweed *Eucheuma cottonii* and provided by a commercial source. Gelatins from different starting materials were obtained from Croda Colloids, UK and Sigma, UK. The biopolymers used are listed in table 2-1 below.

Crude Bovine Protease was obtained from Sigma.UK, whereas beta-mannanase was obtained from Megazyme, Ireland. All other chemicals were obtained from Sigma, UK.

	Type	Supplier
Gelatin	Type B, IEPpH 5	Croda & Sigma
Carrageenan	Type 1, Kappa	Sigma
Locust bean gum	Crude extract	Industrial source

Table 2-1 Biopolymers used in this study.

2.2. Sample preparation

2.2.1. Phosphate buffer

Two buffers were used in this study. A phosphate buffer at pH 6.8 was made by dissolving 4.595g $Na_2HPO_4.12H_2O$, 1.561g KH_2PO_4, 2.923g NaCl and 0.500g KCl in 1l deionised water. A second buffer was made from 2% $K_4P_2O_7$ (tetra-potassium pyrophosphate or TKPP), 0.05% NaCl 0.05% KCl and made up to 1l in deionised water (w/v). This was adjusted to pH 7 using HCl at 25°C.

Calculation of the ionic strength was performed using the equilibrium constants for each ionised group formed. Equations 2.1 to 2.3 give the stepwise ionisation of phosphoric acid and the equlibrium constant obtained from the Handbook of Chemistry and Physics, Student Edition, 1988. In a similar manner pyrophosphate ionises as detailed in equations 2.4 to 2.7, which also give the equlibrium constants. The equlibrium constants are used to calculate the proportion, α, of each ionisable species at a given pH, using equations 2-5 to 2-9. Using this information and equation 2-8 it is possible to arrive at the theoretical ionic strength value, μ. The phosphate buffer (pH 6.8) had an ionic strength of 0.116M whereas the TKPP buffer was at an ionic strength of 0.564M.

$$H_3PO_4 \leftrightarrow H^+ + H_2PO_4^-, \quad K_{a1} = 7.52\times10^{-3} = \frac{[H^+][H_2PO_4^-]}{[H_3PO_4]}$$ Equation 2-1

$$H_2PO_4^- \leftrightarrow H^+ + HPO_4^{2-}, \quad K_{a2} = 6.23\times10^{-8} = \frac{[H^+][HPO_4^{2-}]}{[H_2PO_4^-]}$$ Equation 2-2

$$HPO_4^{2-} \leftrightarrow H^+ + PO_4^{3-}, \quad K_{a3} = 2.2\times10^{-13} = \frac{[H^+][PO_4^{3-}]}{[HPO_4^{2-}]}$$ Equation 2-3

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$$H_4P_2O_7 \leftrightarrow H^+ + H_3P_2O_7^-, \quad K_{a1} = 1.40\times10^{-1} = \frac{[H^+][H_3P_2O_7^-]}{[H_4P_2O_7]}$$ Equation 2-4

$$H_3P_2O_7^- \leftrightarrow H^+ + H_2P_2O_7^{2-}, \quad K_{a2} = 3.20\times10^{-2} = \frac{[H^+][H_2P_2O_7^{2-}]}{[H_3P_2O_7^-]}$$ Equation 2-5

$$H_2P_2O_7^{2-} \leftrightarrow H^+ + HP_2O_7^{3-}, \quad K_{a3} = 1.70\times10^{-6} = \frac{[H^+][HP_2O_7^{3-}]}{[H_2P_2O_7^{2-}]}$$ Equation 2-6

$$HP_2O_7^{3-} \leftrightarrow H^+ + P_2O_7^{4-}, \quad K_{a4} = 6.0\times10^{-9} = \frac{[H^+][P_2O_7^{4-}]}{[HP_2O_7^{3-}]}$$ Equation 2-7

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$$\alpha_0 = \frac{[H^+]^3}{[H^+]^3 + K_{a1}[H^+]^2 + K_{a1}K_{a2}[H^+] + K_{a1}K_{a2}K_{a3}}$$ Equation 2-8

$$\alpha_1 = \frac{K_{a1}[H^+]^2}{[H^+]^3 + K_{a1}[H^+]^2 + K_{a1}K_{a2}[H^+] + K_{a1}K_{a2}K_{a3}}$$ Equation 2-9

$$\alpha_2 = \frac{K_{a1}K_{a2}[H^+]}{[H^+]^3 + K_{a1}[H^+]^2 + K_{a1}K_{a2}[H^+] + K_{a1}K_{a2}K_{a3}}$$ Equation 2-10

$$\alpha_3 = \frac{K_{a1}K_{a2}K_{a3}}{[H^+]^3 + K_{a1}[H^+]^2 + K_{a1}K_{a2}[H^+] + K_{a1}K_{a2}K_{a3}}$$ Equation 2-11

$$\mu = 0.5\sum C_i Z_i^2$$ Equation 2-12

2.2.2. Sample dispersion

Dispersion of the biopolymers was achieved by adding powder to buffer where a vortex had been achieved using a submersible magnetic stirrer and follower. Where dispersion was difficult, a more vigorous vortex was achieved using a Stuart Scientific SS2 motor driven impeller or an Ultraturrax T-25 homogeniser.

2.2.3. Purification of locust bean gum

Locust bean gum (LBG) solutions were prepared by suspending industrial grade LBG in buffer at either 1% or 0.5% w/w. These were heated for 30 minutes at 80°C and centrifuged for 20 minutes at 1000 x g using a Multex benchtop centrifuge to remove insoluble material. The dissolved material was calculated as 0.58% per 1% crude powder used, correcting for 10% moisture.

In some cases a further purification was achieved using ethanol precipitation. Four volumes absolute ethanol was mixed with 1 volume centrifuged cLBG solution. The insoluble biopolymer was collected after 2 hours, dried overnight and ground to a fine powder. This material was resolubilised using the same procedure as in 2.2.2.

2.2.4. Preparation of biopolymer mixtures

Carrageenan and gelatin were hydrated with buffer using the sample dispersion method 2.2.2. The amount of buffer used for the hydration represented the remainder from that which was used to prepare the LBG solution, 2.2.3, on a % w/w basis.

2.2.4.1. Order of addition

The LBG solution was added to the hydrated gelatin and carrageenan mixture and the mixture heated to 90°C, to solubilise the carrageenan, and then held at 80°C for 30 minutes.

2.2.5. Autoclave treatment of mixtures

Autoclaving was conducted using an MC-25 Benchtop Autoclave from Rodwell Instruments, Basildon, UK. Autoclave times varied between 1 and 60 minutes at 121°C using a programmable control unit. Chamber temperature was measured using a thermo-couple in a virtual sample of the same size as the sample vessels. The sample vessels used were 100ml Schott bottles capable of withstanding autoclaving. Heating and cooling regimes were constant at 30 minutes for each.

2.2.6. Extraction of water soluble component of meat emulsion used in pet food manufacture

Emulsified turkey necks were mixed with an equal mass of pH 6.8 phosphate buffer. This mixture (100g) was placed in autoclave resistant Shott bottles and heated for 15 minutes at 121°C. The cooled mixture was centrifuged (1000 x g) and the supernatant recovered and filtered using a vacuum through Whatman No 4 filter paper. Protein determination was conducted on the supernatant using the Lowry protein determination detailed in section 2.3.3.2. To achieve a concentration of 2.5% in the extract the supernatant was partially dried overnight at 105°C.

2.3. Composition of industrial product and characteristics of the components

2.3.1. Charge measurement

Surface charge measurement using polyelectrolyte titration was conducted using a Mütek PCD 03 pH particle charge detector supplied by Carisbrooke Instruments, Surrey, UK. Dried sample was solubilised in deionised water and titrated against 0.001N polyelectrolyte. Negatively charged samples were titrated against the polycation polydimethyl diallyl ammonium chloride (poly-DADMAC). Positively charged samples were titrated against the polyanion polyethene sodium sulfonate (pes-Na). The samples were diluted in 25ml of deionised water and were placed in the PTFE measuring cell. Titrations were performed using the 702 SM Titrino, Metrohm, Switzerland and PC based Mütek application software. Charge per gram active substance was calculated using equation 2.13. V is consumed titrant volume (l), c is titrant concentration ($eq.l^{-1}$), wt is active substance of sample (g) and q specific charge quantity ($eq.g^{-1}$).

$$q = \frac{V \times c}{wt}$$ Equation 2-13

2.3.1.1. Principle of streaming potential method

The method relies on the adsorption of charged particles onto a PTFE cell surface by Van der Waals forces. The action of a piston inside the cell generates shearing forces. These forces remove part of the associated counter-ions and this is measured as a streaming potential in mV. This value itself cannot be used directly to measure charge (Bley 1992), due to factors such as viscosity and particle size. Charge is measured by titrating against the streaming potential using an oppositely charged polyelectrolyte. The equalisation of charge is dependent on a 1:1 stochiometry (Bley 1992).

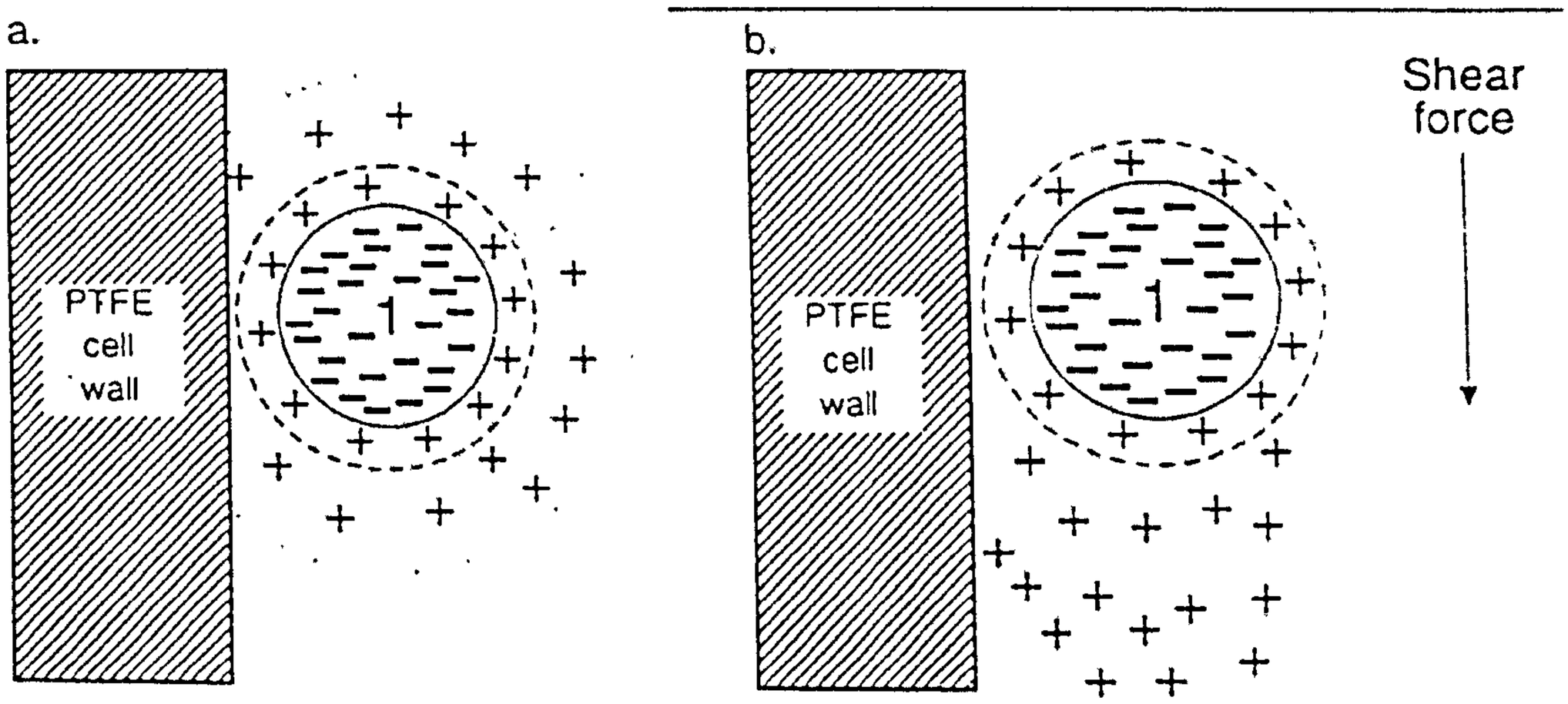


Figure 2-1 The behaviour of charged particles in the Mütek test cell under no shear (a) and under shear (b).

2.3.2. Isoelectric point determination

Isoelectric point is defined as the point where a molecule has no net charge (Christian 1980) i.e. the combined positive and negative charge balance each other out rather than the absence of charge.

Isoelectric point measurements were performed using the Mütek PCD 03 pH as detailed above. Titration end-point was determined as the pH at which a zero shearing potential was achieved. Titrations were performed manually using 0.1M HCl or NaOH. Sample concentrations of between 0.1 and 1% were used to allow precise estimation of the isoelectric point value.

2.3.3. Protein determination

2.3.3.1. Dumas method

Protein determination by the Dumas method was carried out by International Laboratory Services, Shardlow, UK. Samples were combusted in a furnace at 1150°C and the combustion gases collected in a ballast tank after passing through a selection of filters. Further filtration resulted in separation of helium and N_2, which flowed to one side of a conductivity cell, with the carrier gas on the other side. The gases on both sides of the thermal conductivity cell were compared and a voltage output was generated from which the nitrogen was estimated. This method measured all soluble and insoluble protein as well as non-protein derived nitrogen. Protein percentages were estimated as 5.55 x total nitrogen measured. The figure 5.55 was selected on the basis that type B gelatin has been found to contain a nitrogen content of 18% (Eastoe and Leach 1958).

2.3.3.2. Lowry method

Estimation of soluble protein was carried out using a protein assay kit from Sigma Diagnostics, UK (P 5656). Kit contents were as follows: modified Lowry reagent (L 1013), Folin and Clolcalteu's phenol reagent (F 9252) and bovine serum albumin (BSA) protein standard (P 7656). A standard curve was prepared using diluted protein standard solution (400 $\mu g.ml^{-1}$) to give protein concentrations of 50, 100, 200, 300 and 400 $\mu g.ml^{-1}$. Using a 4 ml cuvette, 1.0 ml Lowry reagent was added to 1.0 ml of protein standards, water blank and samples. The cuvettes were left for 20 minutes. To each cuvette 0.5 ml of Folin and Clolcalteu's phenol reagent was added with immediate mixing and left to allow colour development for a further 30 minutes. 2.5 ml deionised water was added to each tube before reading the absorbance values at 750 nm. Having subtracted the blank value, protein concentration was calculated using a standard curve generated using Curve Expert 1.34 curve fitting software.

2.3.4. Hydroxyproline determination

Hydroxyproline determination was carried out by International Laboratory Services, Shardlow, UK. Samples (4g) were hydrolysed in 30 ml 3M H_2SO_4 at 105°C for 16 hours. The hot hydrolysate was filtered through a Whatman No 4 filter paper, residue was washed using hot 3M H_2SO_4 and added to the hydrolysate.The filtrate was cooled and diluted with distilled water. The hydrolysate was further diluted to achieve a hydroxproline concentration within the range 0.5 $\mu g.ml^{-1}$ to 2.0 $\mu g.ml^{-1}$. Chloramine-T (2ml, sodium N-chloro-p-toluenesulfonamide trihydrate) solution (1.41 % w/v) was mixed with 4.0 ml aliquots of the diluted hydrolysate and allowed to stand at ambient for 20 minutes. A blank was prepared substituting distilled water for the diluted hydrolysate. L-hydroxyproline standard solutions were prepared by dissolving 100 mg L-hydroxyproline in distilled water diluted to 200 ml after adding 2 drops of 3M H_2SO_4, this was further diluted 5 ml in 500 ml. Volumes of the diluted hydroxyproline solution of 10, 20, 30 and 40 ml were made up to 100 ml with distilled water to give four standard solutions of 0.5, 1.0, 1.5 and 2.0 $\mu g.ml^{-1}$. Colour reagent was prepared by dissolving 10.0 g 4-dimethylaminobenzaldehyde in 35 ml perchloric acid solution and slowly adding 65 ml of propan-2-ol. Using capped tubes

2.0 ml of colour reagent was added to 4.0 ml of the standard solutions, blank and sample, mixed thoroughly and heated at 60C in a water bath for 20 minutes. After cooling the tubes by running cold water, these were left for 30 minutes before transferring the contents to 1 cm cuvettes and measuring absorbance at 558 nm and subtracting the blank value. Hydroxproline concentrations were calculated using the gradient from the standard curve and dividing by dilution factors.

2.3.5. Amino acid composition

2.3.5.1. Total amino acids

Determination of amino acids was carried out by Masterfoods, UK. Hydrolysis of sample is achieved by refluxing samples with 6M HCl for 23 hours. Nor-leucine was used as an internal standard at the start of hydrolysis. After pH adjustment, the solution was diluted with loading buffer and loaded onto an LKB amino-acid analyser. The individual amino acids were separated by ion-exchange chromatography and quantified by comparison with the internal standard. This assay allowed the quantitative determination of the following amino acids: aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, methionine, iso-leucine, leucine, tyrosine, phenylalanine, histidine, lysine and arginine. Amino acid values reported here use a recovery factor based on the summation of all individual amino acids as a percentage of the crude nitrogen * 6.25. The acceptable range for this recovery is 85-110%. An alternative factor of 5.55 was found to give more accurate recovery.

2.3.5.2. Cystine and methionine by performic acid oxidation

During the process of acid hydrolysis cystine and methionine are partially degraded. Therefore performic acid oxidation is used. Oxidation converts the unstable cystine and methionine into the stable derivatives cysteic acid and methionine sulfoxide/methionine sulfone. After this stage the samples are neutralised and subjected to the acid hydrolysis procedure 2.3.5.1.

2.3.6. Total Sugar Analysis

Sugar analysis was carried by the Division of Nutritional Biochemistry at the University of Nottingham. Neutral sugars were measured using gas liquid chromatography (GLC) as alditol acetates following hydrolysis by sulfuric acid described by Englyst, Wiggins et al. 1982. For the galactomannan samples, approximately 20 mg was dissolved in 25ml of 1M H_2SO_4 and boiled at 100C for 2h. The preparation of the alditol acetates is given in the above reference. GLC analysis was carried out by injecting 0.5µl of sample into a BPX70 column (0.32 mm Ø 30 m long), SGE Ltd, UK. The flame ionisation detector, Pye 104 GC, was set at 200°C with a flow rate of 5ml.min^{-1}.

2.4. The effect of phase separation and gelatin on turbidity, viscosity and gel-strength of non-autoclaved mixtures of carrageenan and locust bean gum

2.4.1. Turbidity studies

The use of turbidimetric measurements to indicate the onset of phase separation has been used recently by Antonov and Goncalves 1999 in studies of gelatin and kappa carrageenan in different aqueous environments. Turbidity was assessed at a wavelength of 490 nm in this study, which was the one used by the above authors. Gelled mixtures of carrageenan, cLBG solution and gelatin were prepared according to 2.2.4., poured into 4 ml plastic cuvettes and allowed to cool at ambient temperature. Using an LKB Biochrom Ultrospec 4050 spectrophotometer, absorbancy values were recorded. The cloud point (or point at which the sample macroscopically phase separated) was regarded as the midpoint of two concentrations of gelatin between which an increase in absorbancy value of more than 2-3% was observed.

2.4.2. Rheological determination

2.4.2.1. Oscillation studies

The estimation of phase behaviour in the samples by oscillatory measurement was conducted using a Bohlin CS-10 controlled stress rheometer. This was fitted with a modified C-14 cup and bob (internal Ø 14mm, external Ø 15.5mm), which had ribbed surfaces to eliminate wall slip due to syneresis, a common problem in carrageenan gel rheology (Hermansson 1989). Measurements were made at a frequency of 1Hz and a strain of 0.01, which was in the linear viscoelastic region (LVER).

2.4.2.2. Determination of linear viscoelastic region (LVER)

LVER is determined by applying increasing sinusoidal amplitude (strain) to a sample and measuring the stress response. Below a critical amplitude the ratio of stress to strain is constant. This is the LVER. A strain of 0.01 was selected for comparison of samples as this was in the LVER.

2.4.2.3. Determination of gelation and melting temperature

Samples were subjected to temperature ramps at heating/cooling rates of 1°C.min^{-1}. Measurements were conducted in the following order: cooling from 80 to 25°C, frequency sweep between 0.01 and 10Hz, heating from 25 to 90°C. The mechanical spectra were used to determine the elastic modulus, G', at 25°C. The temperature where tanδ = 1 was taken as the gelation/melting point.

2.4.2.4. Viscosity studies

Viscometric analysis of samples was conducted using a Bohlin CVO 50 instrument in controlled rate mode. The CVO 50 controls displacement by calculating the stress necessary to achieve a given shear rate. The ability of the instrument to control rate is dependent on the sample and sensitivity of the geometry. The geometries used were a highly sensitive 40/50 (internal/external diameter in mm) double-gap and a 4°/40mm cone, both stainless steel.

2.5. The concentration and molecular size dependence of gelatin on the rheology of the mixtures

2.5.1. Viscoelastic measurement

Estimation of the magnitude of the elastic modulus, G', and the melting temperature were conducted in accordance with methods in section 2.3.

2.5.2. Breakstrength determination

Measurements were conducted using a TA-XT2i texture analyser with a 5kg load cell from Stable Micro Systems, UK. A delrin bloom probe was used with a tapered edge. This had a diameter of 12.7mm. Forces were measured under compression and in return to start mode. Other conditions were: test speed = 0.5mm/s, trigger force = 0.05N, penetration depth = 7mm. From the force/distance spectra the peak force at rupture was taken as the breakstrength. Power law transformations of the spectra provided an index of strain hardening. The sample geometry of 37.5mm ∅, 10mm thickness was achieved by casting in perspex moulds with cover slips.

2.5.3. Experimental design software

It was decided to conduct a three-factor experiment using an experimental design software package called Design Expert, from the Stat-Ease Corporation, Minneapolis. A central-composite, response surface design was selected, as this type of design would allow relationship between the factors to be quantified. The factors selected were: curing time between 30 and 120 minutes, curing temperature between 5 and 30°C and gelatin concentration between 1 and 4%. The data from 14 samples was entered into the model. Design Expert provides linear, quadratic and cubic fits to the data provided, and statistically evaluates them and the individual model terms giving probability of significance or F values. These show the likelihood of the model being attributed to a genuine signal from the data.

2.6. The protective role of gelatin during thermal processing

Results indicated that removal of the different components after processing would help elucidate the nature of any thermal-protective mechanisms in the mixture. This was achieved by enzyme degradation to determine the effect on viscosity of different parts of the mixture. These results are given in chapter 5.

2.6.1. Enzymatic digestion of mixtures

Gelatin, carrageenan and LBG mixtures in pH7 phosphate buffer (100ml), before and after autoclaving, were incubated at 50°C overnight with 25µl β-mannanase (11.25 units). This was then diluted 1:1 with buffer and to it was added 7.3 units bovine pancreatic protease before being further incubated overnight at 50°C. Sodium azide at 0.05% was added to the mixture prior to digestion to prevent microbial growth.

2.6.2. Viscometric measurement of mixtures

As described in section 2.4.1.4., viscosity was measured using a double-gap geometry at a temperature of 50°C unless otherwise stated. The viscosity value was selected from the lowest shear rate, $1s^{-1}$ in the case of the non-enzyme digested samples. In the case of the low viscosity enzyme-digested samples a shear rate of $6s^{-1}$ was selected. This value was in the low shear Newtonian plateau, at lower shear rates there was considerable noise in the data due to measuring system insensitivity.

2.6.3. Determination of molecular weight of carrageenan SEC-MALLS

The molecular weight measurements were conducted using a Waters 590 HPLC pump, with TSK 5000 & 6000 separating columns at 60°C. This was connected to a Dawn DSP laser photometer and Wyatt/Optilab 903 inferometric refractometer supplied by Wyatt Technology, USA. The running buffer was pH 6.8, µ=0.1 solutions were pre-filtered through 0.45µm membrane filter.

Samples were prepared by heating 1.5mg/ml of carrageenan in 0.1M LiCl at 90°C for 15minutes. Prior to measurement the samples were held at 60°C. Before injection the samples were filtered through 0.45µm membrane filter. The filtered samples were loaded into the heated chamber to commence injection.

Results were analysed using the Astra 4.20 software also from the Wyatt Corporation, Santa Barbara, USA. Average molecular weight values were obtained by selecting the region attributed to signal from the elution profiles. A dn/dc estimate of 0.15 ml/g was used for LBG and 0.188 ml/g for gelatin. The dn/dc is how much the refractive index of a solution varies for a given increment in concentration.

2.6.4. Breakstrength

Breakstrength conducted as described in section 2.5.2.

This concludes the materials and methods chapter; the next three chapters detail the results obtained using the methods described above.

3. Composition of industrial product and characteristics of the components

3.1. Introduction

In section 1.2 of the introduction it was explained that in the manufacture of some wet meat products, polysaccharides are used to form a gel or thickening agent in which meat based proteins are suspended. There have been observations that gels from final products do not have the properties predicted from studying the behaviour of the polysaccharides. An obvious difference between the product and gel studies is the presence of the meaty chunk. The question was therefore: what component or property of the chunk was responsible for the change in gel strength? It was considered that the most likely component to be leached from the chunks would be the water-soluble gelatin as it is formed from the collagen.

Initial work therefore had to be carried out to establish:

- ❖ The nature and amount of gelatin leached from meat chunks and if there were differences in its composition and amount due to different types of raw material and processing regimes.
- ❖ Suitable polysaccharides for the work also had to be established. The commercial materials used can be very variable.
- ❖ The behaviour of the system will also be dependent on other materials present. In the factory polyphosphates were used and their influence of the complex system needed to be evaluated.

The purpose of work described in this chapter was to establish the components and processing conditions in the industrial manufacture so that materials and experimental methods can be adopted for use in systematic investigations.

3.2. Estimation of protein amount and type from retorted samples containing two meat sources and two pack designs.

To establish the amounts of protein coming from industrial samples, a study was undertaken to analyse the soluble proteins released during the thermal processing of products cooked in the absence of the gelling polysaccharides. Two different meat sources were used: a bonecake material and one derived from animal heads and feet. Two processing conditions were selected to give equivalent lethality, F_0, based on thermal processing calculations that are used by the manufactures for their can and pouch systems.

The pouch was made from a sealed foil laminate containing 100g sample. This was heated to 125°C for 25 minutes after a come-up time of 15 minutes and then cooled to 30°C over 15 minutes. Contents of the cans weighed 390g and were heated to 121°C over 10 minutes, held at 121°C for 51 minutes, cooled to 40°C over 10 minutes then held at 40°C for 28 minutes.

After processing and before analysis cans and pouches were heated to melt out any gelatin type material. The contents were filtered using cheese-cloth and the filtrate collected for analysis. The filtrate was analysed for total solids, total protein using Dumas method and hydroxyproline content (see section 2.3.3). Dried samples were sent to Masterfoods for amino acid analysis and nitrogen levels were also established (methods given in section 2.3.5).

3.2.1. Protein and hyroxyproline content

Table 3-1 shows the results of analysis of the two meat sources cooked in can and pouch. The liquids drained from the meaty chunks contained about 5% solids. By converting the nitrogen levels to protein using a factor of 5.55 it would appear that most of the solid material is protein, between 68 to 75%. What is of interest is the relevant amounts of gelatin in the samples. Hydroxyproline assays were carried out on the wet extract as these imino acids only occur in connective tissue. Typically they constitute 10% of the total weight of gelatin; therefore multiplication of their weight by 10 will give the amount of gelatin in the wet sample (Eastoe and Leach 1958). As

shown in table 3.1 the gelatin levels in the wet extract are about 2% and hence half of all the protein present.

The total processing time in the cans is greater than for the pouches and this may explain the higher gelatin levels seen in the canned samples. Heads and feet also seem to yield more gelatin than the bonecake.

	N %	Protein % (N x 5.55)	Hdxy %	Gelatin % (Hdxy x 10)	Ratio (Gel%/P%)	Dried soilds %	% protein in dried sample
Can, Heads and Feet	0.75	4.16	0.28	2.76	0.66	5.93	70.2
Pouch, Heads and Feet	0.71	3.94	0.25	2.53	0.64	5.22	75.5
Can, Bonecake	0.69	3.83	0.22	2.18	0.57	5.60	68.4
Pouch, Bonecake	0.62	3.44	0.19	1.86	0.54	4.72	72.9

Table 3-1 Nitrogen (N determined by Dumas method) and hydroxyproline (Hdxy) values for bonecake and heads and feet materials processed in can and pouch. Dried is % solids after 3 days at 80°C. Nitrogen and hydroxyproline values are for a single analysis performed by International Laboratory Services, UK.

3.2.2. Amino acid composition

As well as the amount of protein present, the amino composition was considered to be important as it helped establish the gelatin to other proteins ratio. The amino acid composition of the gelatin could be an important variable as the sources from which it was derived varied.

Amino acid analysis of the 4 industrial samples was conducted in liaison with Masterfoods, and the commercial company International Laboratory Services provided data on the hydroxyproline content. The different amino acids are often grouped according to their functional categories.

Valine, isoleucine, leucine and phenylalanine form the aliphatic group.

Aspartic acid and glutamic acid form the negative group.

Histidine, arginine and lysine form the positive group

Cysteine, threonine, tyrosine, methionine, serine and alanine form the other group.

Glycine, proline and hydroxyamino acids (the majority of which will be hydroxyproline) are given separately.

Table 3-2 shows the results from analysis of the different samples. For comparison, the amino acid composition of ox skin and bone derived collagen is given, (Eastoe and Leach 1958). For completeness, the data for the hydroxyproline is included. The values obtained for the wet extract were calculated from the dried material, the same sample, which was used for the amino acid determination. The dried sample nitrogen content was determined using the sum of all the individual amino acids to give a percentage for the crude nitrogen, and the values obtained are included in the table.

The total proteins estimated for the powders is about 10% lower than those stated in Table 3-1. This could be due to the samples being analysed for amino acid content having picked up moisture. Thus the % protein appeared lower.

The proportion of gelatin in table 3-1 indicates that the bonecake and heads and feet includes non-gelatin protein. It is possible to estimate the proportion of gelatin present by using the glycine and proline/hydroxyproline proportions. Typically these account for a third and a fifth, respectively, of the total amino acids in gelatin (Eastoe and Leach 1958). However, in the extract produced glycine and proline compositions include a non-gelatin component. Their increased concentration, as was found for the hydroxyproline, in heads and feet compared to the bonecakes is suggestive of more gelatin being derived from this material. It is less clear that the additional processing of the cans had any effect.

	Percentage amino acids of total protein in dried sample.				‡ Published data (per 100 residues)	
	Can, heads and Feet	Pouch, heads and Feet	Can, Bonecake	Pouch, Bonecake	Ox skin	Ox bone
glycine	19.25	19.65	17.29	17.44	33.50	31.40
proline	10.47	10.94	9.16	9.53	11.86	11.88
hydroxy	†7.28	†7.77	†6.76	†6.94	9.92	10.72
aliphatic	12.03	11.44	13.10	12.57	7.37	7.77
negative	20.08	20.04	21.79	21.82	12.09	12.56
positive	14.80	15.07	15.79	16.10	7.47	8.10
other	16.09	15.09	16.11	15.60	17.73	17.52
% protein in dried sample (N*5.55)	63.6	64.2	60.6	58.2	-	-

Table 3-2 Amino acid composition for bonecake and heads and feet materials processed in can and pouch. Expressed as a percentage of all amino acids in the dried sample.

† Data obtained by direct hydroxyproline analysis of native collagens.

‡ (Eastoe and Leach 1958).

The four samples showed small variations in the proportion of charged amino acids. Large differences in the proportion of charges may be important in determining interactions with other charged components. The table 3-2 shows that the nature of the protein is different from typical gelatin in that the amount of charged groups are increased. Whether this is important is less clear, as many of the charged amino acids will be associated with globular proteins and extracted myosin that may have leached from the meat samples, but may not be at the surface of the molecules so that they can interact with other macromolecules. Charge on proteins can be estimated from their amino acid composition and this has been carried out. The effective charge at the surface of the molecules or aggregates of the denatured materials can be very different from that of the theoretical calculated values.

Charged amino acids play an important role in the determination of total charge exhibited by the protein. The total charge is the sum of all the negative and positive functional groups and terminal amino and carboxyl groups. The buffer pH is the single most important factor in determining charge in proteins as this determines the proportion of each dissociated group. Charge values are therefore meaningless unless the pH at which the measurements are undertaken is also given. In the case of the four canned and pouched samples charge values, as measured by a streaming potential (section 2.3.1), at pH 3.0, 6.8 and 10.0 are given in table 3-3 along with the iso-electric point (IEPpH). Charge values below IEPpH show a positive value. In the four

samples this was observed at pH 3.0. Higher positive values are possible where there is a greater proliferation of dissociated amide groups and a reduction in the number of carboxyl groups. Principally, this can be related to the amino acid composition and the numbers of the respective charged residues. The amount of charge is dependent on the measurement pH and the IEPpH. Despite the finding that the heads and feet had more gelatin present, i.e. less charged proteins, little difference in the amounts of charge is seen between the samples.

	pH	3.0	6.8	10.0	IEPpH
Charge ($\mu eq.g^{-1}$)	Can HF	49.5	-31.7	-76.4	4.2
	Pouch HF	59.1	-31.6	-92.2	4.5
	Can BC	40.5	-32.2	-77.8	3.7
	Pouch BC	98.2	-31.4	-86.1	4.2

Table 3-3 Charge determination values in micro-equivalents per gram of dried extract and iso-electric point data for the four samples using the Mütek Particle Charge detector. HF denotes heads and feet, BC denotes bonecake.

Figure 3-1 shows charge values over a pH range for two types of Sigma gelatin. These values are in accord with data obtained for gelatin using the streaming potential method (Faassen 1991). These values show an approximate 3-fold reduction in the charge of the meat extracts at pH 6.8 compared to the pure gelatin. This reduction is due to the different proteins present and possibly a reduction in functionality during thermal processing. The presence of different proteins will cause differences in the total IEPpH. Myosin is a common meat protein and has an IEPpH of ~5.5.

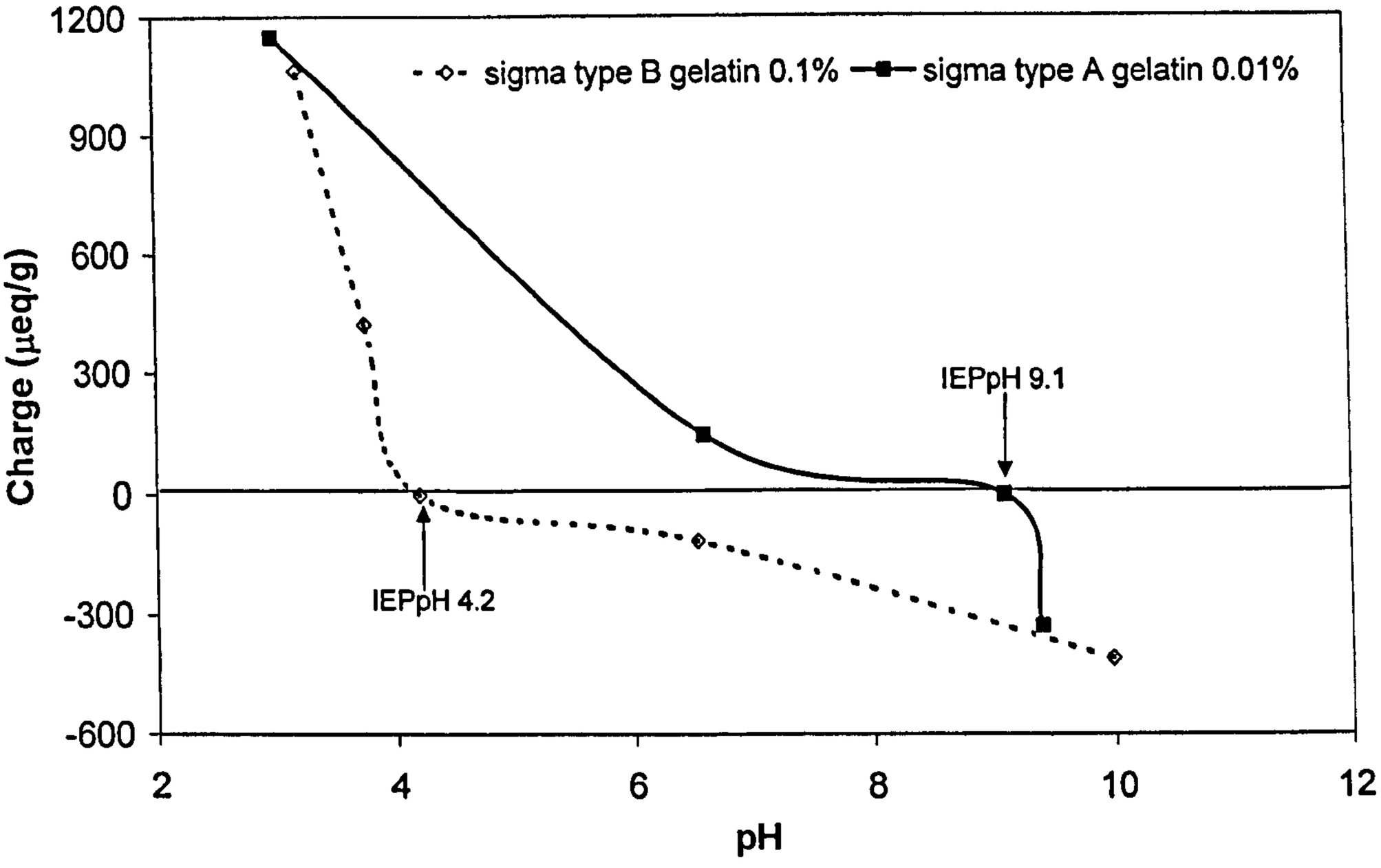


Figure 3-1 Polyelectrolyte titrations of Sigma type B (0.1%) and Sigma type a (0.01%) gelatins.

3.3. Choice of gelatin

Commercial gelatin can be obtained by liming or acid hydrolysis. The results obtained from the extracts of commercial samples showed that the gelatin in solution would equate most closely to the Type b i.e. limed gelatin. It was decided to source this material either from Croda or Sigma and to test the gelatins in the buffers used to ensure their suitability for further analysis. The concentrations to be studied would be in the range of 0 to 5% so that the values of typically 2.5% seen in the commercial samples for gelatin would be covered. The time profiles for the processing of the gelatin for the experimental work would be similar to those used for the pouch and can sterilisation processes.

3.4. Phosphate interactions with other materials

Observations showed turbidity present in mixtures of Sigma gelatin with the TKPP phosphate buffer. Turbidity is an indicator of phase separation in mixtures of biopolymers, so its presence was undesirable, (i.e. if the mixture was already phase separated, without the inclusion of the polysaccharides, how could any conclusions be made about the phase behaviour when the polysaccharides were present?) Before further studies into the phase separation of biopolymer mixtures were undertaken it was necessary to identify the origins of the turbidity in the gelatin solution alone.

One explanation for turbidity was the formation of insoluble gelatin/phosphate complexes. The polyphosphate TKPP is used in retorted pet food products to improve the water-holding capacity of the meaty chunk and reduce the shrinkage on cooking or "purge". This greater water-holding capacity is achieved by solubilising the meat proteins and increasing the pH, (Townsend and Olsen, 1987). The role of raising the pH and buffering during the cooking process is to decrease rates of polysaccharide thermal degradation, which is an important factor in retorted pet foods. TKPP has the formula $K_4P_2O_7$ and its structure is given in figure 3-2. The dissociation of KO groups provides an overall negative charge for the molecule. One hypothesis was that the TKPP molecule, due to the central -P-O-P-, acted as a bridge between positive side groups on the gelatin polymer. Considerable amounts of phosphate can bind ionically to collagen (Weinstock, King et al. 1967). Biomaterial synthesis of bone substitute utilises the interaction between phosphate and collagen (Du, Cui et al. 2000).

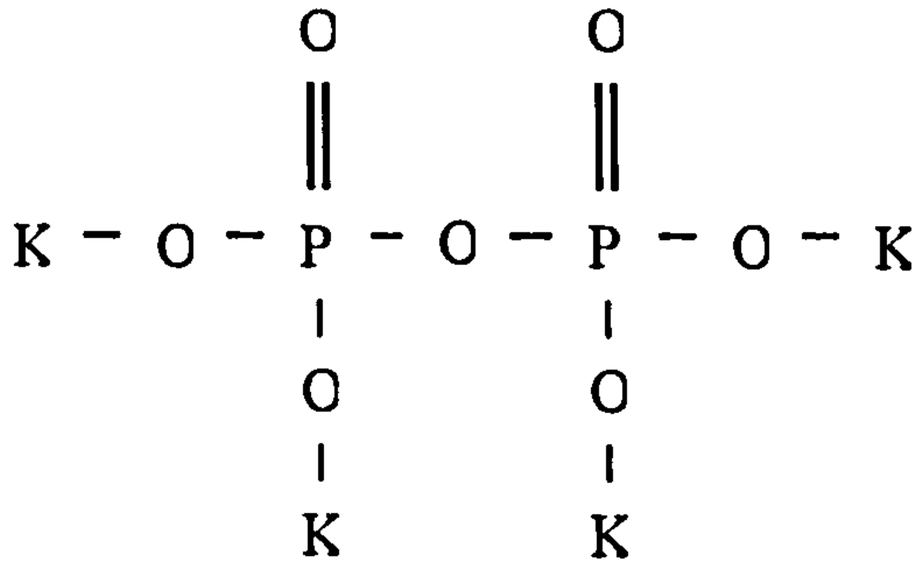


Figure 3-2 Structure of tetra-potassium pyrophosphate.

It was necessary to test the hypothesis that ionic interactions are responsible for turbidity. A 5% solution of Sigma gelatin was dispersed in TKPP buffer. The sample was centrifuged and the supernatant removed. The precipitate was washed in water and re-centrifuged. A portion of the ppt was re-suspended in 1M NaCl to suppress any electrostatic interactions. Visual observation showed no apparent reduction in insoluble precipitate or any other noticeable changes, the sample having a milky appearance. A further sample was subjected to changes in pH by altering the value with concentrated HCl and NaOH. At pH 11.6 no changes were observed, at pH 2.2 the appearance changed from milky white to transparently clear.

To compare with the result for the Sigma gelatin, 5% solutions of Croda 106 and 305 bloom gelatins were prepared in TKPP buffer. No precipitation was observed in the solutions, which remained clear. It was reasoned that either a fundamental difference between the Sigma and Croda gelatins existed, for example different amino acid composition, or there was a non-gelatin component of the Sigma sample, which was responsible for the turbidity.

	Sigma	Croda
glycine	24.07	23.75
proline	13.53	13.54
aliphatic	8.47	8.70
negative	15.30	14.88
positive	12.70	12.48
other	14.18	14.07
total	88.25	87.42
protein (N*5.55)	89.3	88.9

Table 3-4 Amino acid composition of Sigma 225 Bloom limed hide and Croda 305 Bloom limed ossein gelatins.

Amino acid data, as shown in table 3-4, revealed no striking differences between the two samples, certainly none that would explain the visual difference observed. The alternative explanation was therefore investigated (i.e. the precipitate was calcium phosphate). Discussions with Croda Colloids revealed that gelatins can often contain some calcium as a by-product of processing. This is sometimes removed by deionisation. Calcium content is inferred by using conductivity measurement. The conductivity value from the technical data for Croda 305 Bloom was 346 μS (6.67% w/w at 40°C). This had a calcium content of < 100 ppm. In order to test the hypothesis that calcium was the responsible agent, 5% (w/w) Croda limed ossein gelatin with a conductivity of 948 μS (5000 ppm calcium) was taken and mixed with the TKPP buffer. Precipitation was observed as with the Sigma sample. This finding led to the elimination of the Sigma gelatin from further studies involving phosphate buffers.

3.5. Choice of carrageenan

The carrageenan used commercially is not purified. It is known that the quality is variable and it can contain up to 50% other matter. To avoid this variable it was decided to use a known source of material. To help in the decision of the type to use some preliminary work was carried out.

3.5.1. Charge and molecular weight determination in carrageenan

3.5.1.1. Charge

One of the dominant characteristics of carrageenan and an important characteristic in phase behaviour was the charge on the biopolymer. Charge on carrageenan derives from sulfated groups substituted onto the galactose backbone and charge measurement can be used to infer sulfate quantity. The Mutek particle charge analyser provided a sensitive and efficient technique for this measurement.

As a validation of the technique and to give us numbers with which to compare differences between samples, four commercially available carrageenans from Sigma were tested along with a semi-refined sample (SRC) of *Eucheuma cottonii* from Masterfoods. Charge values are shown in table 3-5. There is a comforting similarity between the measured values and those from the literature. The values appear to be within the expected range. The type 1 sample had a similar charge level compared to the pure kappa-carrageenan at a substitution of 0.83 and 0.86 respectively. The data confirms the relative charge per disaccharide for each of the pure types with lambda-carrageenan being the most highly charged.

Comparing the value for SRC with the other kappa types shows it to have a higher level of substitution at 1.07. It may have been assumed that this would be less due to non-carrageenan impurities present in the SRC. However, since samples of carrageenan are often mixtures of the different types, a substitution greater than that for kappa could have been anticipated.

	Measured charge (μeq/g)	Estimated substitution	Hugerth, 1997	Rochas, 1986
SRC	2767	1.07 (770g/mol)	-	-
Type 1	2156	0.83 (770g/mol)	-	-
Kappa	2239	0.86 (770g/mol)	0.96	0.98
Iota	3337	1.55 (928g/mol)	1.49	1.62
Lambda	3918	2.20 (756g/mol)	2.09	1.56

Table 3-5 Comparison of estimated substitution levels per disaccharide for Sigma carrageenans and published values from Hugerth, Caram-Lelham et al. 1997 and Rochas, Lahaye et al. 1986. Numbers in brackets are idealised molecular weight of each disaccharide. Typical CV was less than 1%.

3.5.1.2. Molecular weight

Table 3-6 shows Mw values obtained using light scattering for the samples described in the previous section. Published values for kappa-carrageenan from *Eucheuma cottonii* measured by the same method, were 504 kDa by Singh and Jacobsson 1994 without further treatment, and in LiCl buffer and 487 kDa by Myslabodski, Stancioff et al. 1996 in $LiNO_3$ buffer after dialysis. Our results show a slightly higher value for kappa at 668 kDa with a similar value being recorded for the type 1 sample. Of the other pure samples the lambda sample had the highest Mw at 1411 kDa. Interestingly, the SRC had a higher Mw at 1458 kDa. This may be due to the relative lack of processing this sample received compared with the Sigma samples. An attempt to correlate carrageenan molecular weight with gel breakstrength (data not shown) did not reveal any relationship, so it is unclear what impact this result would have on the gelation properties of the sample. With regard to phase behaviour, the higher molecular weight average would favour phase separation where incompatibility exists.

	Molecular weight (kDa)
SRC	1458 (±207)
Type 1	642 (±65)
Kappa	668 (±76)
Iota	701 (±118)
Lambda	1411 (±250)

Table 3-6 Measured molecular weight values using SEC-MALLS for carrageenans.

For most of the studies caraggeenan sourced from Sigma and quoted as Type 1, predominantly kappa, was used. The amount used commercially is about 0.3% of the total weight of material and 50% of the polysaccharide blend. This formed the basis for the concentration used in the rest of the studies reported in the thesis.

3.6. Choice of locust bean gum

Whilst it was recognised that locust bean gum also contained impurities, it was decided to use industrial LBG after a pre-purification step as described in the methods chapter. The important factor was to remove particulates, and therefore the sample was heated in the appropriate buffer and then centrifuged. The supernatant was added to the other materials. For one particular study an alcohol precipitate of the LBG was used.

3.7. Charge, molecular weight and composition of locust bean gum.

A compositional analysis of a technical grade LBG is in table 3-7 shown from the work of Kok, Hill et al. 1999. The most important features from the perspective of these studies are the levels of particulate and protein, being 34 and 13.5% respectively of the total sample. These are believed to confer a level of natural protection from thermal degradation that will be discussed in Chapter 5. Locust bean gum itself will have no overall charge. It could be possible that the impurities carried some charge, but on measurement the charge values at all pHs were very low (< 5uequ.g^{-1}).

(%w/w)						
Carbohydrate	Protein	Fat	Ash	Moisture	Particulate [b]	Supernatant [b]
72.0	13.5	1.3	2.7	10.5	34	51

Table 3-7 Gross composition of technical grade LBG from (Kok, Hill et al. 1999).

[a] Carbohydate determined by difference

[b] 70°C/h solubilised, 18 500 × g/15 min centrifuged, dried at 105°C in oven.

3.8. Discussion

These results show that gelatin content in products can reasonably be expected to be between 2.0 and 2.5%. The gelatin content within heads and feet material is higher than in bonecake. The most suitable gelatin to use for the studies is that supplied by Croda and the levels of calcium are critical if TKPP buffers are to be used.

Carrageenan will be sourced from Sigma and be predominately kappa Type 1. The LBG used is of a commercial grade, but requires filtration before use and the recognition that it may contain other materials.

Processing procedures carried out at the factory can be matched within the laboratory, and cooking times will affect the levels of gelatin and the molecular weights of all the macromolecules.

Information established from this work was used to define the macromolecules studied for their phase separation behaviour as presented in the following chapter.

4. The effect of gelatin on viscosity and gel-strength of non-autoclaved mixtures of carrageenan and locust bean gum.

4.1. Introduction

As discussed earlier in the thesis, the gel phase of canned petfood products will not only contain the added polysaccharide gelling system, but will also contain proteinaceous components from the meat. The most important of the latter on gel properties is likely to be gelatin formed as a result of the breakdown of collagenous material. Gelatin will be soluble and is likely to have influence on the properties of the system. Of particular interest is phase separation. It has long been recognised that biopolymer mixtures above a concentration threshold will often show segregative phase separation into domains rich in each of the two biopolymers. The objective of the work described in this chapter is to determine if at the concentrations of interest in the product gelatin phase separates from the polysaccharide gelling system. The most important polysaccharide system is carrageenan/LBG, so this work will focus on mixtures of these and gelatin, including the use of purified industrial grade LBG and that obtained from Sigma Chemicals.

Theoretical models describing phase behaviour of synthetic polymer mixtures in organic solvents go some way in assisting our understanding of the major causes of incompatibility in water-soluble biopolymers. The importance of temperature, concentration, solvent affinity and molecular weight are easily transportable ideas between the synthetic and biochemical systems. In relating these ideas to model foods and certainly to real foods, particular problems are encountered. Systems made from industrial starting materials have impurities, which make conclusions based on experimental data more difficult. It can be tempting to attribute observed differences to some factor associated with the pure biopolymer, when in reality the observation was due to an artefact present within the impure starting material. The crude locust bean gum sample used contained non-glactomannan impurities, as has been described in chapter 3. On dissolution at high temperature, which would solubilise the galactomannan, there is still a large quantity of insoluble material.

Kok found that a preparation of technical grade LBG, similar to that used for much of this study, contained 34% particulates (on a wet basis of the original powder) after heating and centrifugation (Kok, Hill et al. 1999). These insoluble particulates can interfere with rheological measurement by acting as fracture initiation sites and lowering measured breakstrength and modulus values. Protein is believed to play a role in protecting LBG from degradation. The proportion present in the industrial sample may be a source of natural protection during autoclaving; this will be discussed in the next chapter. As outlined in the Materials and Methods chapter, the LBG sample used for this work was obtained from the supernatant (i.e. the particulate fraction was removed). Crude preparations are more susceptible to enzymatic degradation due to naturally occurring β-mannanases. Research grade β-mannanase from Megazyme has a temperature optimum of 60°C and is stable below 70°C. In this study the preparation temperature of 80°C was insufficient to completely inactivate the enzyme, and viscosity reduction was observed in solutions stored for 24 hours even when the particulate material had been removed. In this work, after preparation, samples were immediately refrigerated and used within two days where it was shown that viscosity changes were very small.

4.1.1. Phase separation in gelling protein/polysaccharide mixtures

Gelation may itself be considered a phase separation process i.e. the separation of the biopolymer matrix from water. Indeed, visual observations of many gels show them to be more turbid, an indicator of phase separation, than the starting solution. The exception to this is gelatin, which produces clear gels. The melting temperature of a gelling, phase separated, system is dependent on which biopolymer forms the continuous phase. For example the melting temperature of a mixed agar/gelatin gel can vary from 85°C (agar continuous) to 30°C (gelatin continuous), (Brown, Foster et al. 1995). Melting and gelation temperature has been described previously as the G'/G" crossover as defined by (Winter and Chambon 1986). Gelation in a phase causes an infinite increase in its viscosity and imposes a kinetic limit on movement driven by the thermodynamics favouring phase separation. It can be said therefore that gelled systems are not in equilibrium and the extent of phase separation will be dictated by the conditions, which affect gelation, for example cooling rate. A slower

cooling rate may favour more extensive separation. Another phenomenon, which has been reported by Clark, Richardson et al. 1983 in agar/gelatin mixtures, is what is known as osmotic de-swelling. In this case two gelled, phase separated, networks transfer water by osmosis from the agar to the gelatin causing the agar to de-swell.

4.2. Turbidimetry

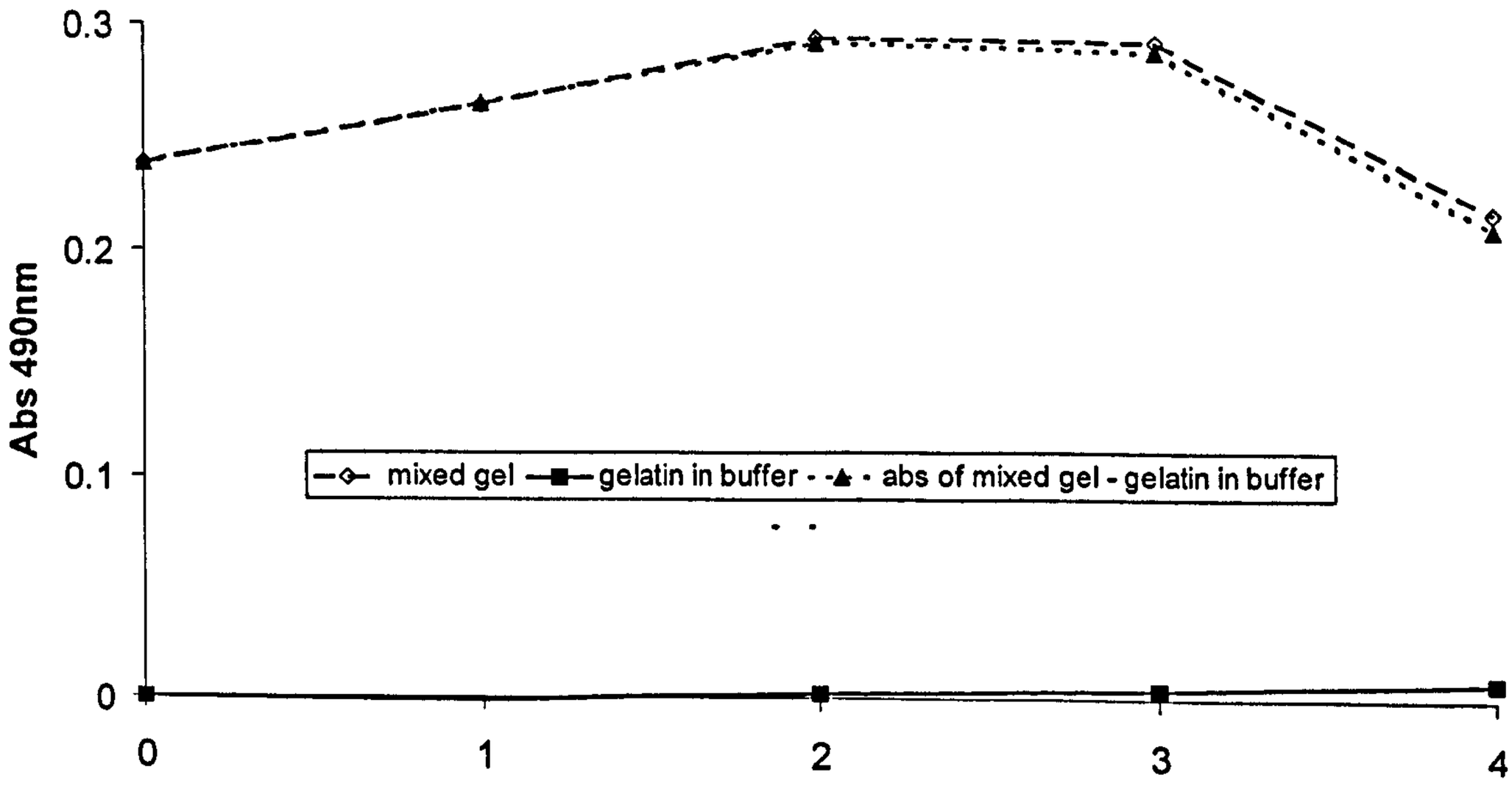


Figure 4-1 Absorbance 490nm of mixed gel of carrageenan 0.3% and LBG 0.3% with Croda 305 gelatin, compared to gelatin gel alone. Phosphate pH 6.8 buffer. At ambient temperature.

Figure 4-1 shows relative absorbance values for carrageenan and locust bean gum mixtures with increasing gelatin concentration. The figure also shows the relative absorbance of gelatin in the phosphate pH6.8 buffer alone. This curve shows little increase in absorbance with increasing gelatin as would be expected. The relative absorbencies for the mixed gel show a marked increase at concentrations up to 2%, with a maximum at some point between 2 and 3%, before the values fall again at 4%. This can be explained by a gradual increase in phase-separated micro-domains up to a critical gelatin concentration. Between 2 and 3% a limit is reached in the amount of absorbance in the sample. Above 3% the sample separates macroscopically into precipitated aggregated domains. The gel, in which these domains are suspended, consists of dissolved polymers. These regions have a very low absorbance.

4.3. Rheology

Rheological methods can be used to observe phase separation in mixtures of two or more biopolymers. The response can be compared with what would be predicted for a homogenous mixture. Where departure from predictions has been observed it is important to remove the possibility of causes other than phase separation. An example of this may be where a viscosity increase is observed where one biopolymer is added to another biopolymer solution, resulting in a total concentration which then exceeds the critical coil overlap concentration C* (Morris 1984).

In phase separated mixtures time dependent rheology (e.g. thixotrophy) is often observed. Gelatin solutions show Newtonian flow behaviour (Wulansari, Mitchell et al. 1998) whereas LBG solutions are highly shear thinning. Time dependent rheology is never observed in LBG solutions (Alves, Antonov et al. 1999).

The studies in this section used Sigma locust bean gum and Croda 305 Bloom gelatin that were dissolved using pH6.8 phosphate buffer.

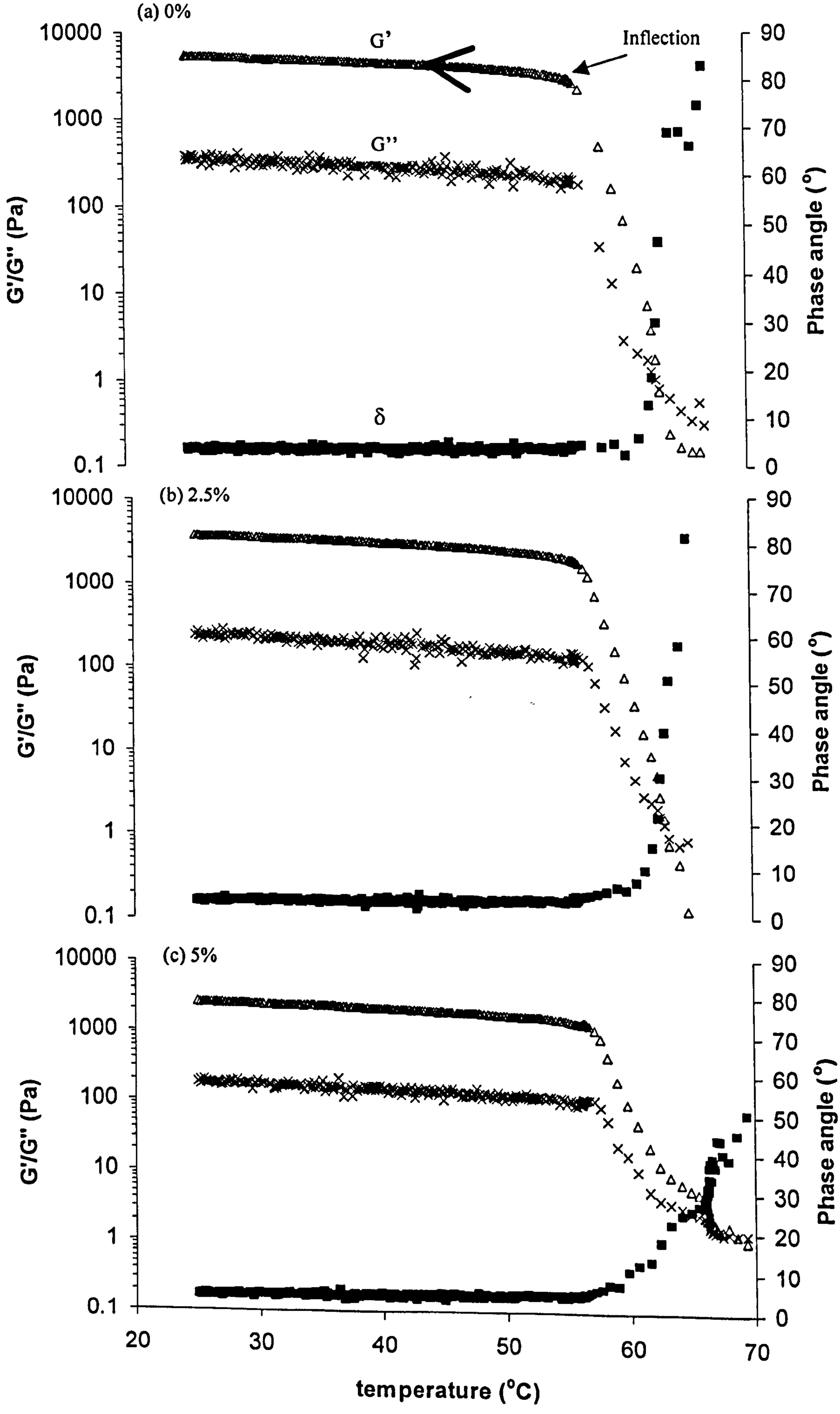
4.3.1. Investigation of the effect of gelatin on the small deformation behaviour of gelled mixtures

Figures 4-2 and 4-3 show respectively the cooling and heating responses of carrageenan and locust bean gum mixtures with varying gelatin concentration. In figure 4-2 at 0% gelatin the temperature of G'/G'' crossover (phase angle $\delta = 45°$), which can be taken as the gelation temperature on cooling, was 63.0°C. The respective values for systems containing 2.5, 5, 7.5 and 10% gelatin were 63.1, 66.9, 69.2 and 66.9°C. At 0% gelatin the temperature at which there was an inflection in G' from the rapid increase in G' to a plateau was 56.2°C (see fig 4-2a). The respective values for gelatin at 2.5, 5, 7.5 and 10% were 56.1, 56.5, 56.8 and 57.0°C. Similarly, in figure 4-3 the G'/G'' crossover temperatures on heating for the gelatin concentrations were 88.9 and 88.4°C for 0 and 2.5% respectively, the subsequent values >90°C. The shape of the melting curve did not yield such a sharp inflection, but it was possible to take the intercept temperatures from two extrapolated lines from the two linear parts of the curve, see 4-3a. The respective values were 81.6, 79.7, 77.3, 76.1 and 78.4°C. Figures 4-4a and 4-4b show the combined elastic moduli for the cooling and heating runs.

The melting and gelation temperatures of the mixture show there is no fundamental change in the systems structure as the gelatin concentration is increased. The dominant phase has the gelation temperature of a carrageenan/LBG continuous phase; there is no evidence of phase inversion. This is in accord with Kelly, who showed carrageenan/LBG melting temperature to be constant up to and including 20% gelatin (Kelly 1995). This contrasts with the agarose/gelatin system where at gelatin concentrations between 5 and 10% in the presence of 1% agarose, there is a decrease in melting temperature from 85 to 30°C, (Brown, Foster et al. 1995). Agarose is uncharged and does not have the charge-entropy effect promoting miscibility as opposed to carrageenan.

Increasing gelatin concentration has an effect on the shape of the sol/gel transition, with particular reference to the phase angle. At 0 and 2.5% gelatin the sol/gel transition is smooth; at subsequent gelatin concentrations there is the appearance of a shoulder in the gelation spectra, see figure 4-2. In figure 4-3a the response of the

elastic modulus to cooling shows a broadening of the transition part of the curve as the gelatin concentration increases.



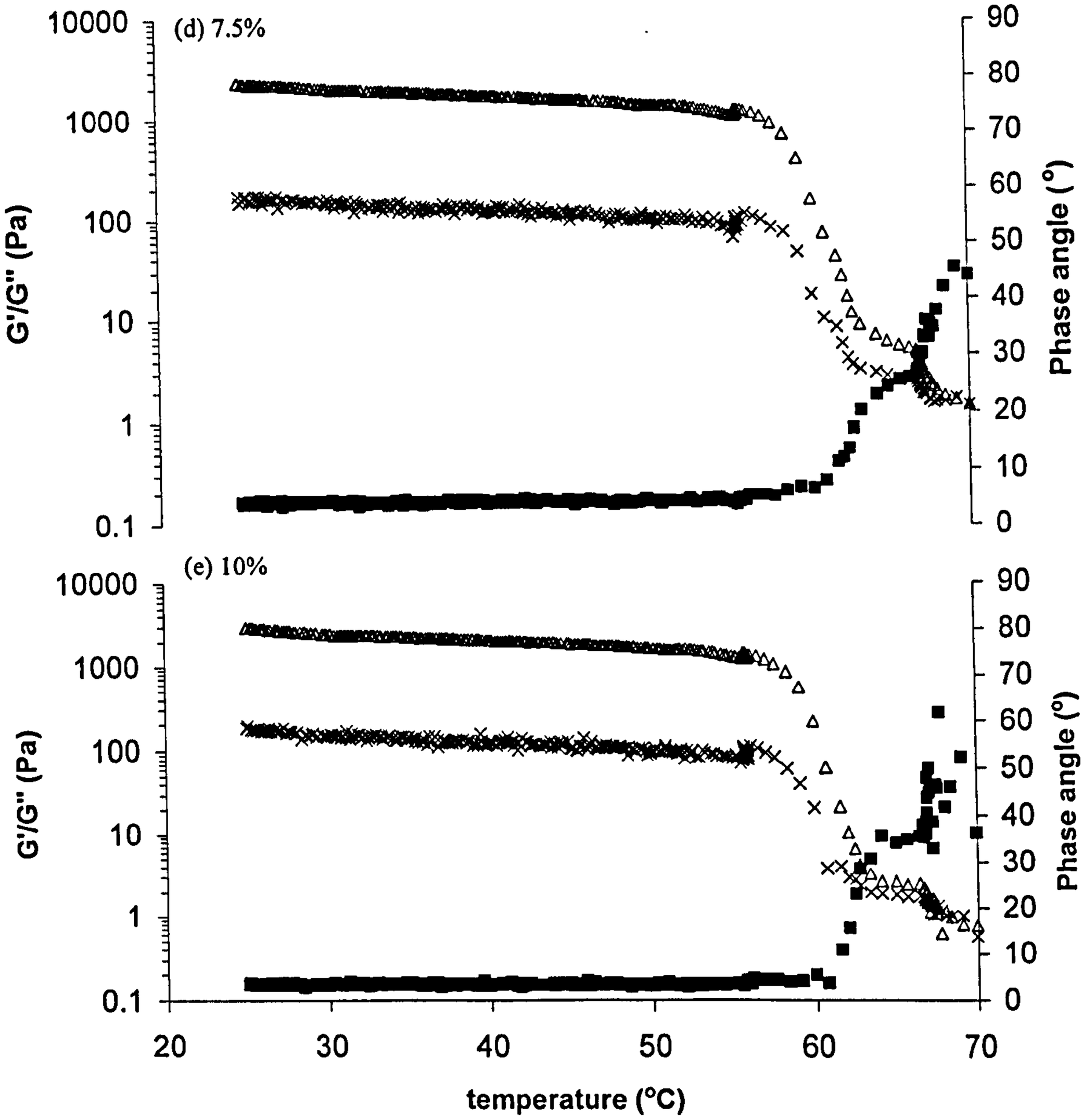
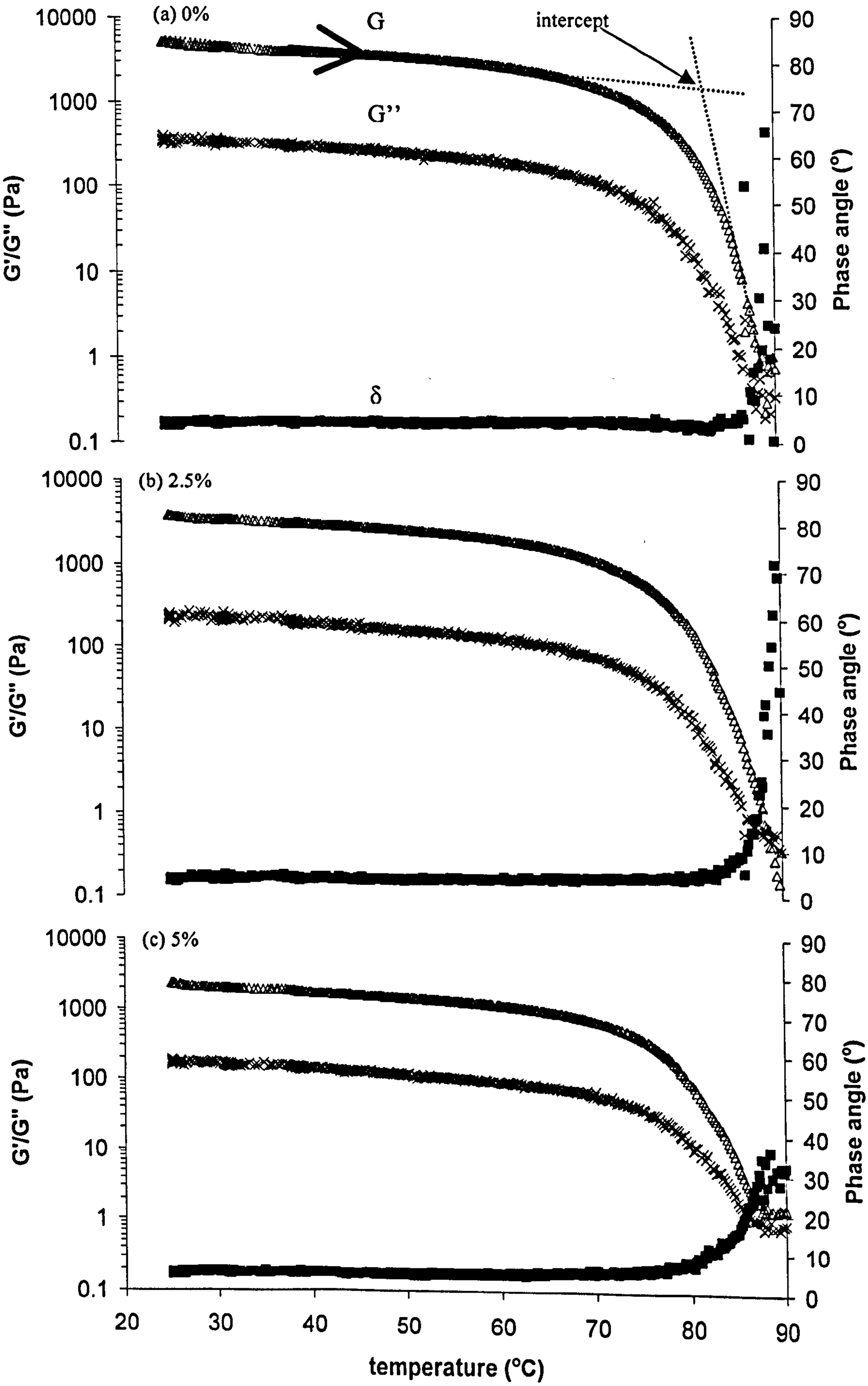


Figure 4-2 Cooling curves for 0.3% carrageenan, 0.3% LBG (see 3.2.3) and gelatin at concentrations (a) 0%, (b) 2.5%, (c) 5%, (d) 7.5%, (e) 10% w/w. Heating rate was 1.5°C/minute. Viscoelastic parameters displayed are G' - Δ, G'' – x and δ - ■.


(a) 0%
G
intercept
G''
δ
(b) 2.5%
(c) 5%
G'/G'' (Pa)
Phase angle (°)
temperature (°C)
10000
1000
100
10
1
0.1
90
80
70
60
50
40
30
20
10
0
20
30
40
50
60
70
80
90

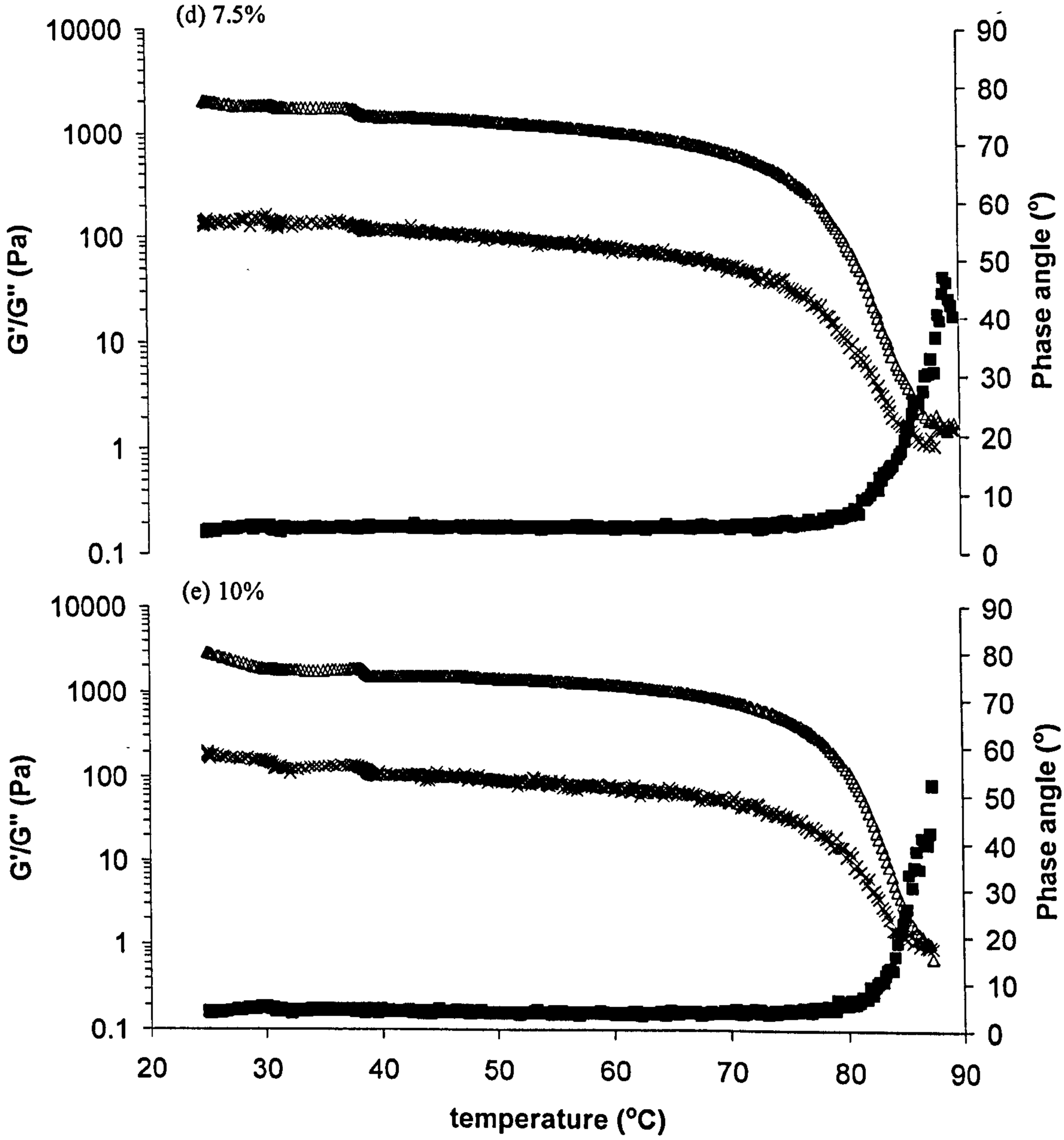


Figure 4-3 Heating curves for 0.3% carrageenan, 0.3% LBG (see 3.2.3) and gelatin at concentrations (a) 0%, (b) 2.5%, (c) 5%, (d) 7.5%, (e) 10% w/w. Cooling rate was 1.5°C/minute. Viscoelastic parameters displayed are G' - Δ, G'' – x and δ - ■.

The effect of adding gelatin can be best demonstrated by figure 4-4. The initial values at 25°C of G' on cooling from figure 4-2a were 5.41, 3.73, 2.51, 2.32 and 2.98 kPa respectively with increasing gelatin concentrations. This shows that gelatin causes gel weakening at concentrations up to 7.5%. Above this value the extent of gel weakening is less with a recovery of the elastic modulus being observed. At 10% gelatin it is possible to see a further part of the cooling/heating curve which only appears at temperatures below 30°C. This component causes a further increase in G', above that attributable to thermal curing of the carrageenan/LBG network, and is consistent with the idea that the gelatin is able to contribute to the strength of the polysaccharide network.

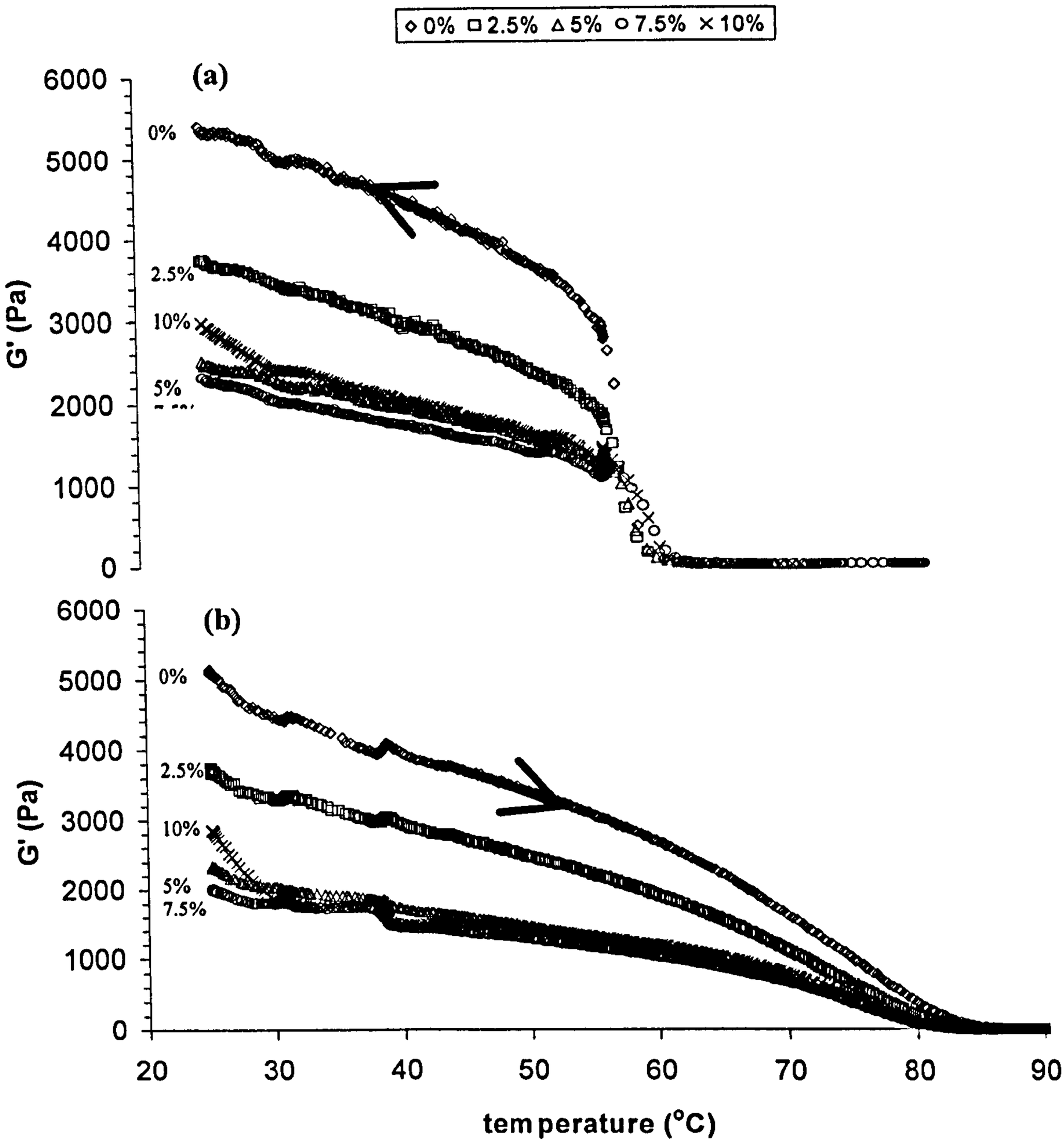


Figure 4-4 Elastic moduli in (a) cooling and (b) heating for mixtures represented in figure 4-2.

4.3.2. Investigation of the effect of gelatin concentration and curing time and temperature on large deformation behaviour of gelled mixtures

This section is concerned with the effect of gelatin concentration and curing time and temperature on gel strength in large deformation. An experimental design approach was employed for some of the work presented. The work presented was mostly conducted using the TA-XT2i Texture Analyser from Stable Micro Systems.

Figure 4-5 shows a comparison of small and large deformation measurements conducted on the 0.3% carrageenan and 0.3% LBG mixtures with increasing gelatin concentration. Small deformation measurements were performed at 25°C and the values shown are at time zero after cooling from 80°C. The breakstrength measurements were performed after curing the gels for 18 hours at 5°C. It can be seen that the addition of gelatin causes a reduction in modulus from 5.41kPa at 0% gelatin to a minimum of 2.32 at 7.5%. At 10% gelatin there is a slight increase in modulus to 2.98kPa. This reduction in modulus is consistent with the idea of gel weakening due to phase separation. The breakstrength data shows an increase in gel strength with increasing gelatin concentration from 3.28N at 0% gelatin and 4.02N at 2.5% gelatin to nearly 25N at 10% gelatin. There was clearly a difference in behaviour of these gels, it was therefore necessary to identify which variable was responsible (i.e. the method of analysis or the curing conditions).

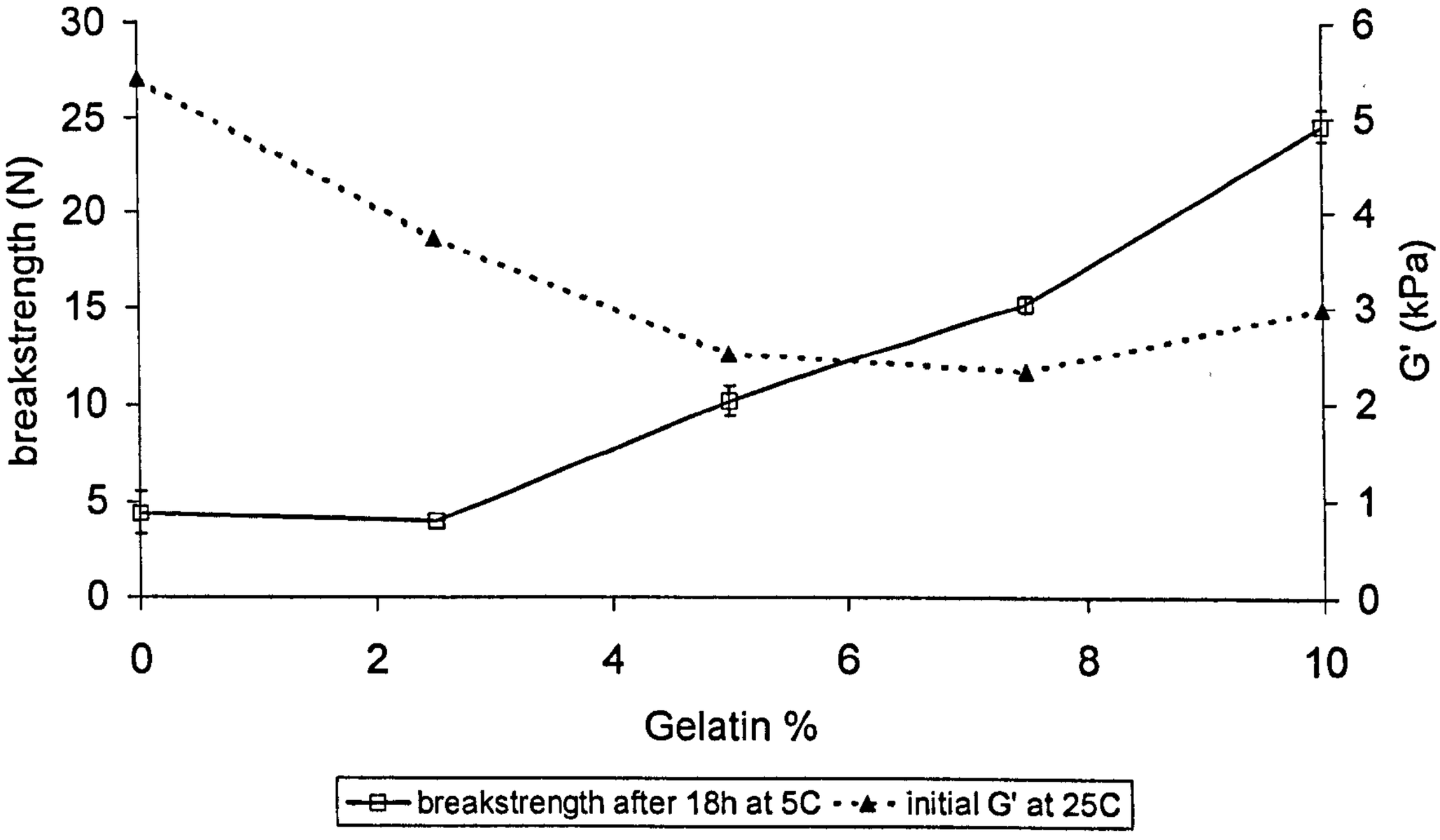


Figure 4-5 Comparison of initial G' at 25°C (dotted line) and breakstrength after 18h at 5°C of 0.3% carrageenan, 0.3% locust bean gum mixtures with increasing gelatin. Error displayed on breakstrength is one standard deviation.

Figure 4-6 shows large deformation data for that previously shown at 18 hours curing time together with a set after 1 hour curing at 5°. Also shown is data from carrageenan/LBG gels at the equivalent polysaccharide concentration as the gelatin containing gels but without the gelatin present. This was to see if any changes in behaviour could be attributed to increases in salt and polysaccharide concentration with the removal of water due to the presence of the gelatin. This data confirms that no such changes were observed with a flat response across the composition range. The data after 1 hour curing time at 5°C shows a different pattern of behaviour to that after 18 hours. From a similar starting value at 0% gelatin of 4.60N, the subsequent gelatin concentrations of 2.5 and 5% show a reduction in gel breakstrength to 1.99 and 3.25N respectively. At 7.5 and 10% gelatin the breakstrength recovers and is higher than the 0% value at 8.18 and 14.0N respectively. The values obtained at 0 and 1% gelatin levels for all treatments reveal less significance of the curing conditions used on carrageenan and LBG gels, in terms of increasing gel strength with time. That is not to say curing time is unimportant, just that at the curing temperature selected here no changes in breakstrength were observed. The response of these gels revealed the necessity to further investigate the effect of curing time and gelatin concentration in determining gel breakstrength.

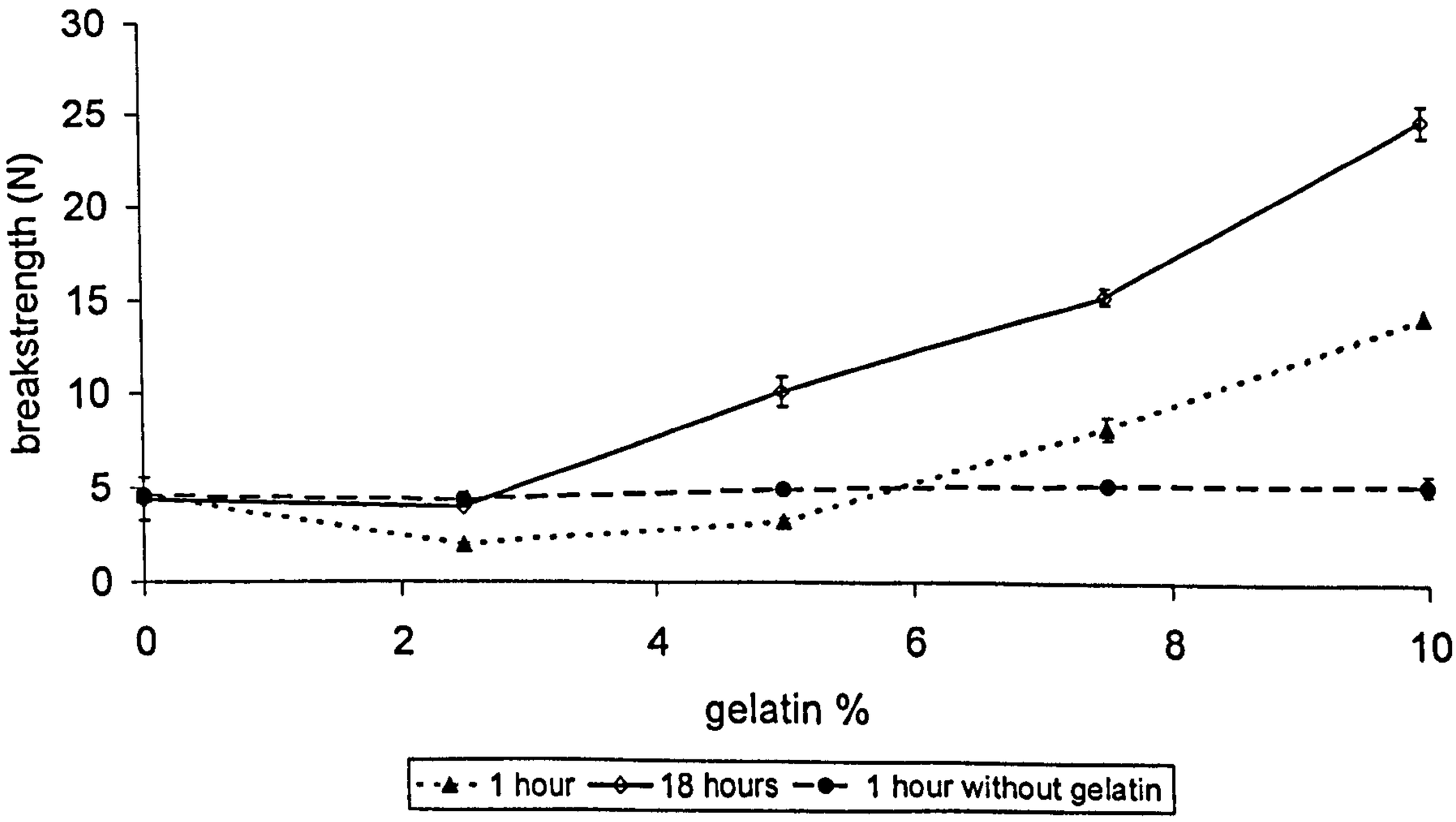


Figure 4-6 Breakstrength of mixtures, 0.3% carrageenan and 0.3% LBG with different levels of gelatin, at 1 hour (dotted line) and 18 hour (solid line) curing times at 5°C. The series denoted by solid circles represents the equivalent buffer concentration, but without the gelatin being present.

Gels were investigated at a narrow range of gelatin concentrations, 0, 1, 2.5, 3.5 and 5%. The curing temperatures selected were 30, 60 and 120 minutes. Figure 4-7 shows breakstrength data obtained for the mixtures at the respective curing times and gelatin concentrations. The data at 0 and 1% gelatin show a similarity in response to curing time at 5°C, the values after 30 minutes being 3.61±1.01N and 4.58±0.36N. Little change was observed over the curing time period for 1% gelatin. At 0% there was a slight increase to 5.29±0.30N. At 2.5% gelatin there was a marked reduction in breakstrength after 30 minutes to 1.43±0.10N. This increased to 2.79±0.25N after 2 hours, which was just over half of that observed at 0% gelatin. Further increasing the gelatin to 3.5% produced a similar response as at 2.5%, but with a lower value after 30 minutes of 0.74±0.07N. At 5% gelatin after 30 minutes curing time the gels had almost the same breakstrength as at 3%. However, after 1 hour there was a marked increase to 3.22±0.07N, after 2 hours the highest breakstrength of any of the samples tested was observed at 5.52±0.20N. This data showed that dramatic breakstrength reductions were observable at relatively small gelatin concentrations. Also, these reductions could be observed at higher gelatin concentration depending on curing time.

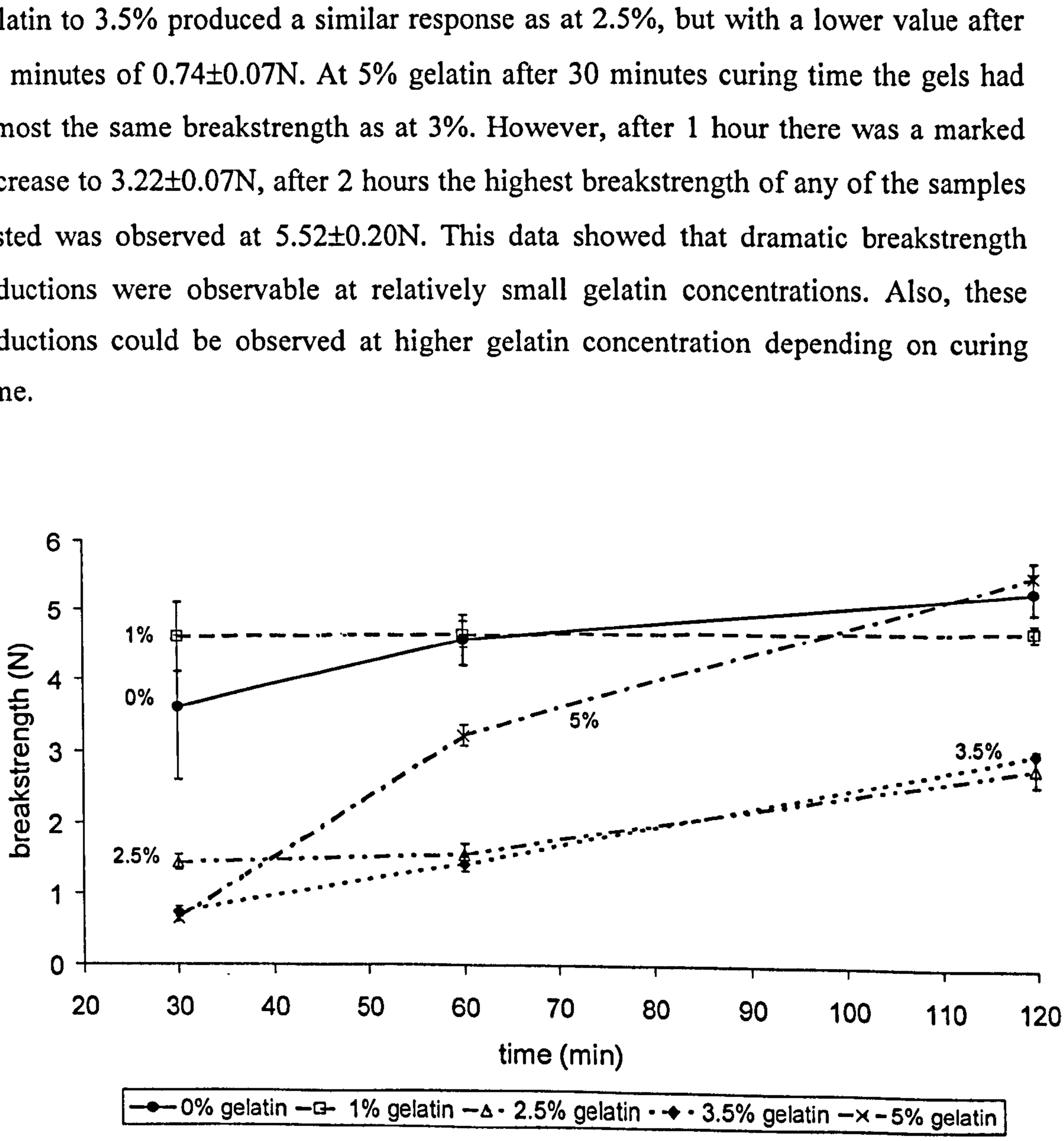


Figure 4-7 Breakstrength of mixtures showing the effect of gelatin on the change in breakstrength over a 2 hour period at 5°C. Error is standard deviation.

All work thus far focused on the relationship between curing time and gelatin concentration. The refrigeration temperature of 5°C was selected for all studies for convenience. However, it was recognised that investigating the effect of curing temperature variation on breakstrength was of interest to the study. Temperature variation was achieved using a cooled incubator that had an accuracy of ±2°C. It was decided to conduct a three factor experiment using an experimental design software package called Design Expert, from the Stat-Ease Corporation, Minneapolis. A central-composite, response surface design was selected, as this type of design would allow relationships between the factors to be quantified. The factors selected were: curing time between 30 and 120 minutes, curing temperature between 5 and 30°C and gelatin concentration between 1 and 4%. The software presented numeric models to explain the relationships between the factors and then statistically appraised the model and the model terms in terms of their significance. The best statistical model gave a quadratic relationship (see equation 4-1), between breakstrength and curing temperature/gelatin concentration. Curing time was rejected as insignificant, which was interesting in itself given previous results. All data were from 75 minutes curing time. All the model terms had probability > F value less than 0.05 or <5% chance that the F value this large occurred due to noise. The larger the F value, the more likely that the variance contributed by the model is significantly larger than random error.

$$Breakstrength(N) = 7.3\times10^{0} - 2.0\times10^{-1}\times T - 2.31\times10^{0}\times c + 4.98\times10^{-3}\times T^{2} + 3.2\times10^{-1}\times c^{2}$$

Equation 4-1 Model of polynomial relationship between gelatin concentration, curing temperature and breakstrength, where (c) is concentration and (T) is temperature. Numbers shown are actual factors.

The model (see equation 4-1) can be represented as a two-dimensional contour plot, see figure 4-9. The lines join regions of the plot where the breakstrength is the same. The plot shows the most unfavourable region is the top-right corner where the gelatin concentration and curing temperature is the highest. Values here are below 1.5N. There is a certain degree of asymmetry in the plot, which identifies gelatin concentration as having a larger influence than curing temperature. Curing temperature is shown to have a more dramatic influence at the higher gelatin concentrations. This is similar to the greater response to curing time at 5°C in figure 4.7. As the concentration decreases the response to curing temperature flattens out.

The extent of change in breakstrength during the process gelatin concentrations and post-process product temperature of approximately 15°C shows a 50% reduction in breakstrength from 2.64 to 1.30N. The actual values generated from this model are lower than previously observed and reflect the significant lack of fit present. This means that the model is unable to accurately predict breakstrength. The lack of fit can be made insignificant by providing more data for the model. This model does, however, provide a picture of the kind of relationship that exists between the curing conditions and gel strength. It is also shows the order of importance of the variables that affect gel strength as: gelatin concentration > curing temperature > curing time.

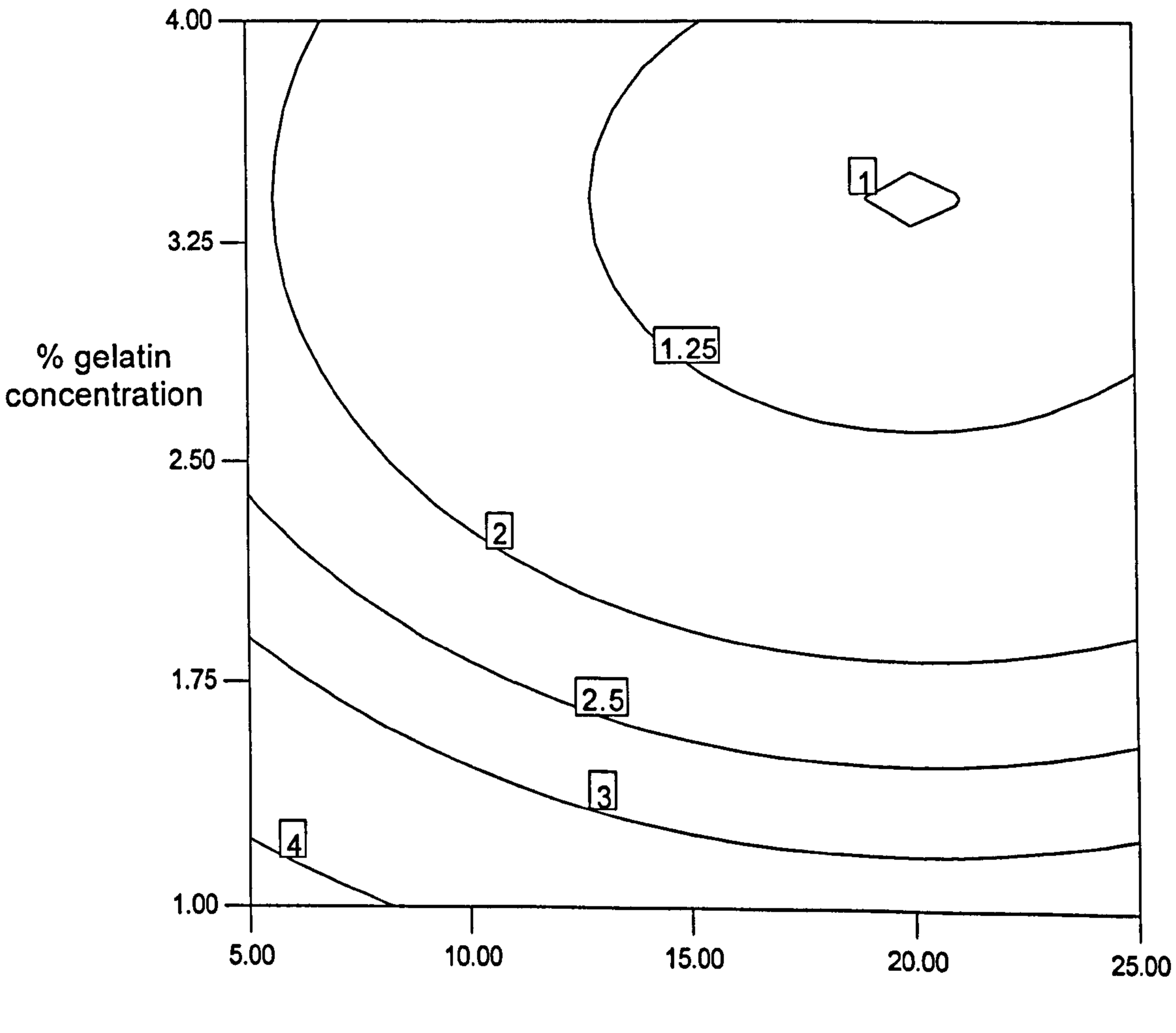


Figure 4-8 Response surface of Design Expert model of breakstrength dependence on curing temperature and gelatin concentration. Breakstrength values (N) at 75 minutes curing time.

4.3.3. Investigation of the viscosity of gelatin, locust bean mixtures and non-gelling lambda carrageenan mixtures

The use of viscometry to further the understanding of the phase behaviour of mixtures of carrageenan, LBG and gelatin presented some issues. Rotational viscometry is a destructive test and cannot be used for gelled samples. The presence of kappa-carrageenan therefore created a problem. It was decided to investigate the viscosity of mixtures of LBG and gelatin in the absence of the carrageenan. A later study used mixtures including the non-gelling lambda-carrageenan to demonstrate the effect of a charged polysaccharide on the phase separation behaviour of these mixtures. Concentrations and temperatures of measurement were selected on the basis of clearly showing differences between mixtures and allowing easier handling of the samples.

The basic premise when dealing with the flow-behaviour of phase-separating biopolymer mixtures is observing the deviation from expected behaviour of non-separating mixtures. One would expect viscosity to increase with concentration regardless of whether you have reached a critical limit or not. Where viscosity decreases with addition of a second biopolymer, some fundamental change in the system has occurred. It is possible to parallel this with precipitation of biopolymers by changing solvent properties using salts or alcohol. Here it is the action of a competing biopolymer which drives one of the biopolymers out of solution.

Viscosity then, can show us whether phase separation has occurred, but can also give information on which biopolymer has formed the dominant phase. This is especially true when the viscosities of the biopolymers are an order of magnitude different at the same concentration.

The material presented in this study shows the flow behaviour of Sigma locust bean gum when combined with Croda 305 limed ossein gelatin in pH 6.8 phosphate buffer.

Figure 4-9 shows the effect of increasing gelatin concentration on solutions of 0.5% LBG. Viscosities were measured between 1 and $100s^{-1}$ at 40°C using a 4°, 40mm stainless steel cone and plate geometry. A measurement integration period, of 120s, was selected to give an accurate value for the viscosity for the samples tested. At 0% gelatin the viscosity of the 0.5% LBG solution has an initial value at $1s^{-1}$ of just above 0.1 Pa.s falling to 0.07 at $100s^{-1}$. This shows the expected pseudoplastic response. At 0.5% the viscosity is of similar values to 0% gelatin across the shear rate range. At gelatin concentrations of 1.0 and 1.5% there is a marked increase in viscosity, above 0.13 Pa.s at $1s^{-1}$. There is also an indication of a steepening of the pseudoplastic response, coupled with more noisy viscosity values. Between 1.5 and 2.0% something drastic occurs and the viscosity falls to a value below that of the 0% gelatin solution. This curve shows a more dramatic decline in viscosity from 0.7 to 0.4 Pa.s across the shear rate range. This fall in viscosity is continued at 2.5% gelatin, which approaches the value of the 2.5% gelatin-only control at 0.03 Pa.s. The curves for the three lowest viscosity samples show an increase in viscosity at the higher shear rates. This was probably due to turbulent, Taylor-vortex flow, and would be expected given the range of sample viscosities and shear rates used. Figure 4-10 shows more clearly the effect of gelatin concentration on mixture viscosity.

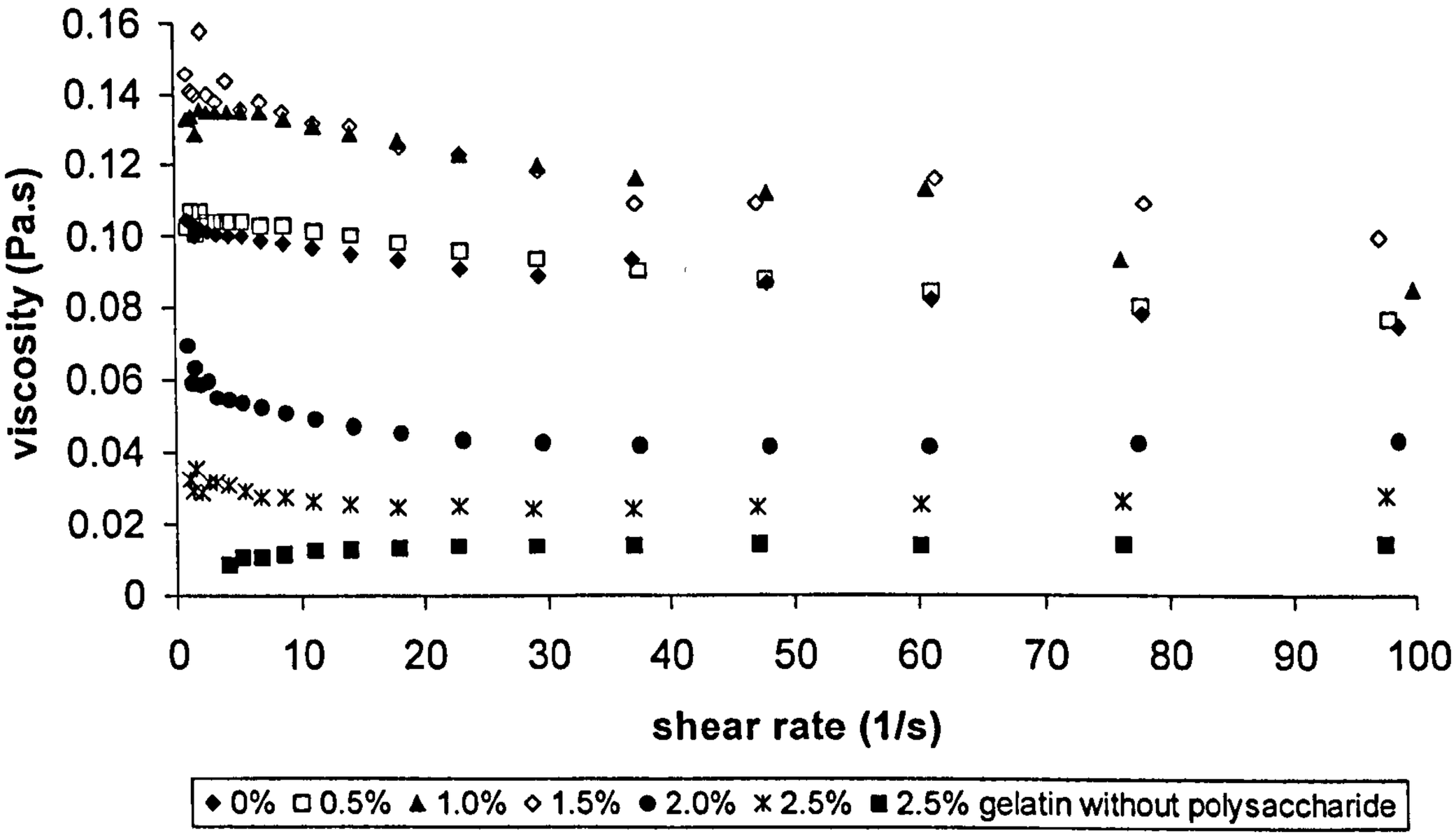


Figure 4-9 The change in viscosity of 0.5% LBG solutions with increasing gelatin concentration. Measurements made 40°C using 4°/40mm cone and plate geometry. Time 0 minutes.

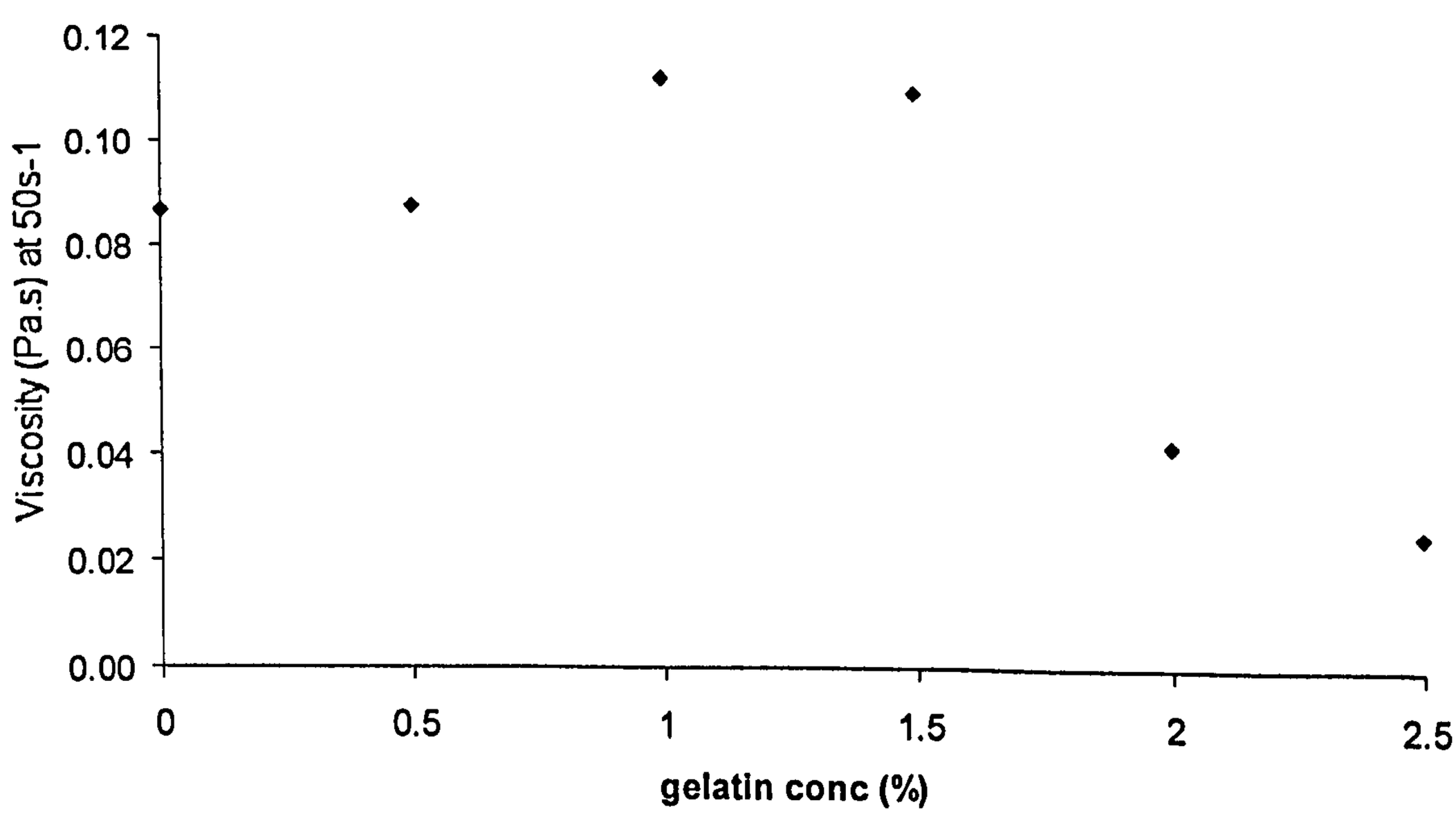
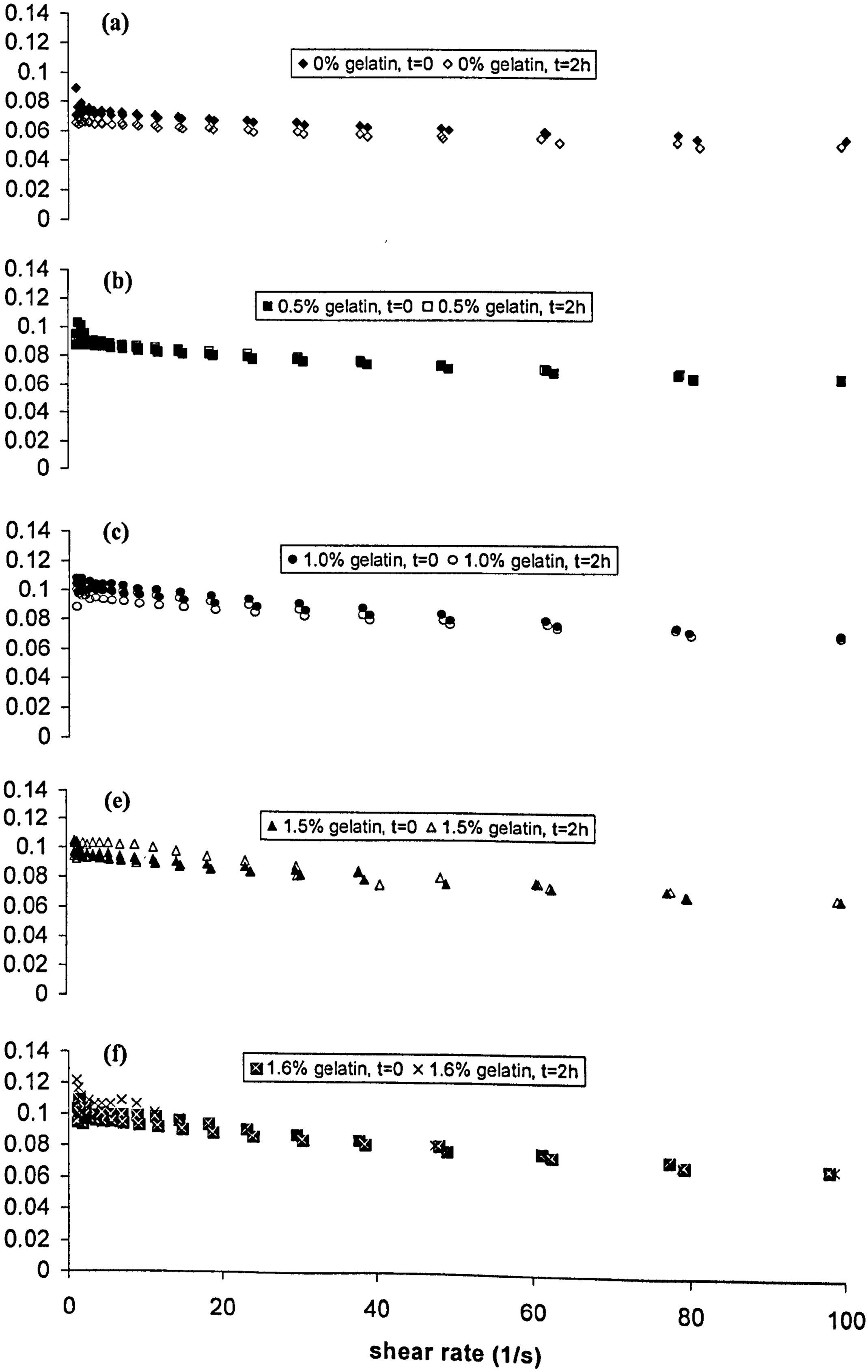


Figure 4-10 Change in viscosity of 0.5% LBG solutions with increasing gelatin concentration shown at $50s^{-1}$.

In order to investigate the phenomena of phase separation induced thinning of the gelatin/LBG mixtures another study was conducted using gelatin concentrations of 0, 0.5, 1.0, 1.5, 1.6, 1.7, 1.8, 1.9 and 2.0%. These were selected to reveal the concentration at which phase separation actually occurred. This time up and down sweeps of shear rate were conducted to reveal sample hysteresis. Hysteresis represents a time-dependent change in flow behaviour not present in pseudoplastic fluids, such as many polysaccharide solutions. Figure 4-11 shows curves of the respective samples at time=0 and after 2 hours standing in a water bath at 40°C. In figures 4-11a and b respective viscosity curves for 0 and 0.5% gelatin at time zero and after 2 hours are shown. The lowest viscosity values displayed are for the 0% gelatin sample between 0.07 and 0.05 Pa.s at t=2h. There is a slight increase at t=0 to between 0.08 and 0.055 Pa.s. The sample at 0.5% gelatin had a viscosity between 0.1 and 0.065 Pa.s, which was consistent for both times. There was no evidence of sample hysteresis. At concentrations of 1.0 and 1.5% gelatin, shown in figures 4-11c and d respectively, a slight viscosity increase above that found at 0.5% is observed. At 1.0% the viscosity ranged from just over 0.1 to 0.07Pa.s across the shear rate range, very similar values were found after 2 hours incubation. At 1.5% gelatin the viscosity ranged from just over 0.1Pa.s to 0.0675Pa.s at time zero, similar values were found after 2 hours. There was evidence of change in flow behaviour after 2 hours for both samples at the lower shear rates with final values being lower than those at the start of the up/down ramp. At 1.7 and 1.8% gelatin there is a decrease in viscosity for the first time in the series. At 1.7% the viscosity ranged from between just under 0.1 to 0.068Pa.s, with the final value of 0.08Pa.s. After 2 hours a similar viscosity response was observed. At 1.8% the viscosity fell from 0.08 to 0.062Pa.s, recovering to 0.071Pa.s at the end of the down ramp. After 2 hours the initial viscosity at $1s^{-1}$ was 0.068Pa.s, this fell to just under 0.06 at $100s^{-1}$ and recovered to 0.073Pa.s at the final shear rate. This change in behaviour was further observed at 1.9% gelatin, at time zero the sample started at 0.09 and fell to 0.056Pa.s before finally recovering to 0.07Pa.s. After 2 hours the viscosity fell from 0.066 to 0.065 and recovered to 0.077Pa.s. The final gelatin concentration, 2.0%, showed the most remarkable response to shear. At time zero hysteresis was clearly observed, figure 4-3j, between 1 and $30s^{-1}$ with the range in viscosity from 0.062 on the up ramp to 0.13Pa.s on the down ramp. The max and min occurred at $4.3s^{-1}$. The initial viscosity value was 0.1 and fell to 0.082Pa.s at $100s^{-1}$ before finally recovering to 0.0775Pa.s at $1s^{-1}$. The viscosity values after 2 hours showed hysteresis

to a lesser extent, but the overall viscosities were much lower. The initial value being 0.068, falling to 0.04 at $100s^{-1}$ and recovering to 0.052Pa.s. The up ramp at 2 hours shows a similarity to the viscosity curve for 2% gelatin in figure 4-10.

Figures 4-9, 4-10 and 4-11 show the change in the viscous response to the addition of gelatin to the LBG solution. It is possible to use the information to indicate at which gelatin concentration phase separation occurs on the basis of viscosity drop. Figure 4-9 shows that separation occurs between 1.5 and 2.0% gelatin. Closer inspection in figure 4-11 seems to show that at 1.7% phase separation occurs. Figure 4-11 also demonstrates the kinetic element in the phase separation process in that the extent of viscosity loss increases with time; this introduced a problem in terms of measuring the mixtures at the same "age" after mixing. Figure 4-11 also showed the responsiveness to shear. In particular, there was a level of recovery in viscosity due to improved miscibility on mixing at the higher shear rates. This is most clearly demonstrated in figure 4-3j. Most importantly the shear history dependence of the phase separating mixtures shows their deviation from pure pseudoplastic flow, which characterises LBG and many other biopolymer solutions. The flow behaviour of LBG solutions is insensitive to shear history (Alves, Antonov et al. 1999). The origin of this shear history dependence is due to the presence of microscopic domains whose size depends on concentration, time and shear conditions. The information provided by figures 4-9 and 4-11 crucially gives us information as to the composition of the continuous phase due to the large difference in viscosity between the gelatin and LBG components of the mixture. At $1s^{-1}$ the viscosity of 0.5% LBG is close to 0.1Pa.s, this compares to a viscosity of 0.03Pa.s for a 2.5% gelatin at the same temperature. The fall in viscosity at concentrations of gelatin approaching 2% and above indicate strongly that it is the LBG that is being driven out of solution as was observed visually. This makes sense thermodynamically, because we would expect counter-ion entropy of gelatin to promote even distribution of the gelatin throughout the mixture. The non-charged LBG has no such requirement. Also, the higher molecular weight of the LBG, as evidenced by its higher viscosity pre-disposes it to be removed from solution more easily than gelatin.


(a)
◆ 0% gelatin, t=0 ◇ 0% gelatin, t=2h
(b)
■ 0.5% gelatin, t=0 □ 0.5% gelatin, t=2h
(c)
● 1.0% gelatin, t=0 ○ 1.0% gelatin, t=2h
(e)
▲ 1.5% gelatin, t=0 △ 1.5% gelatin, t=2h
(f)
⊠ 1.6% gelatin, t=0 × 1.6% gelatin, t=2h
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
20
40
60
80
100
shear rate (1/s)

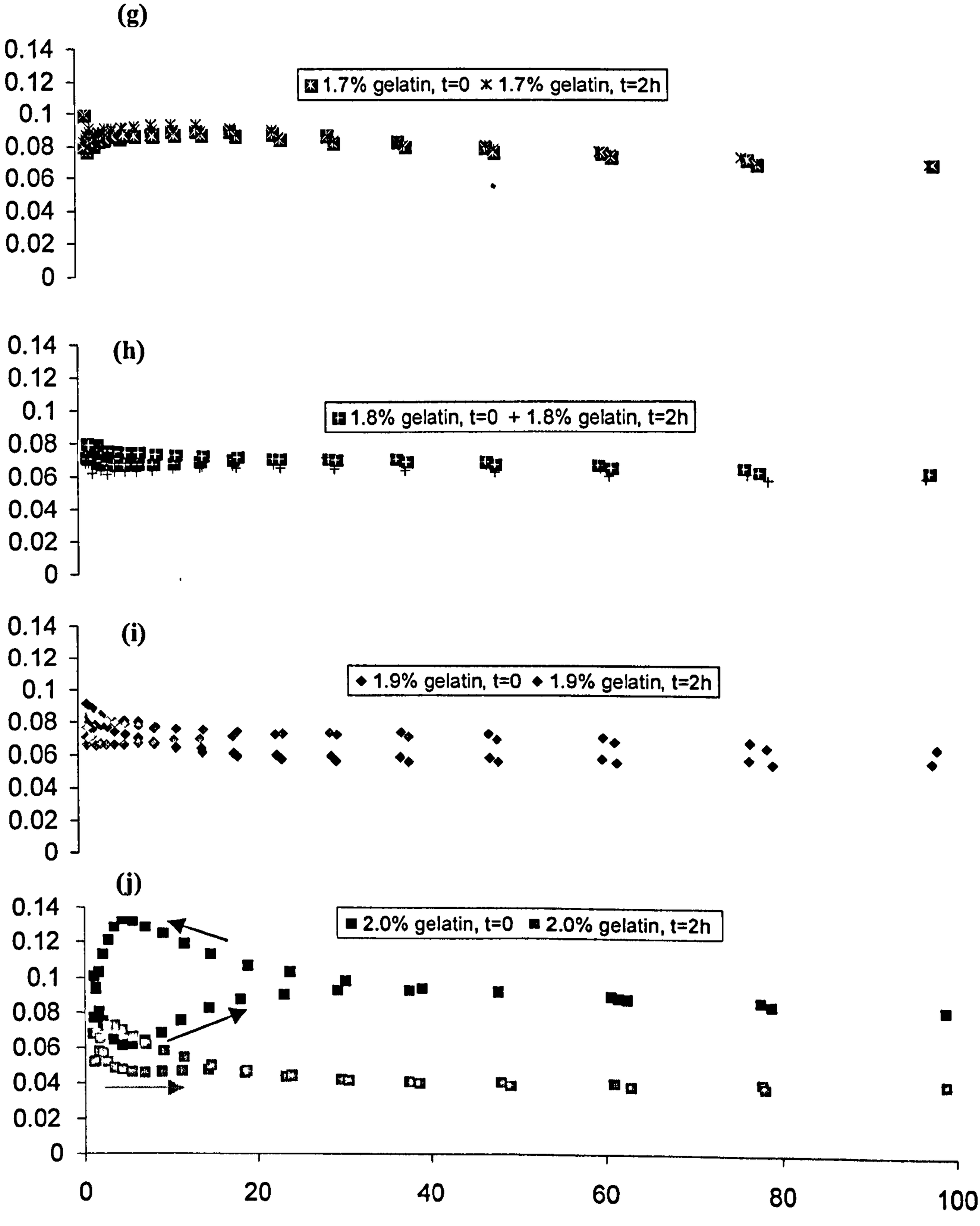


Figure 4-11 The response to flow of 0.5% LBG mixtures with increasing gelatin concentration at time = 0 and after two hours. Measured at 40°C, using 4°/40mm cone and plate geometry.

In order to test the idea of charged biopolymer compatibility, a third, highly negatively charged polysaccharide was added to the mixture in the form of lambda-carrageenan, see figure 4-12. Ratios of λ-carrageenan/LBG at a total concentration of 0.5% were combined with 2.5% gelatin and the viscosity response was observed up to 60 minutes at $1s^{-1}$. The first recorded point was after 2 minutes due to the integration period. At 100% LBG the response over time shows a decrease from 0.03 to 0.02Pa.s after 60 minutes. At 20:80 λ-carr:LBG there is a slight increase in viscosity from 0.04 to 0.0225Pa.s. A further increase is observed at 40:60 λ-carr:LBG from 0.067 to 0.0375Pa.s. This represents the largest drop out of all the samples tested. At ratios of 60:40 and 80:20 the viscosity does not change with time and is constant at about 0.07Pa.s over the time period. At 100% λ-carrageenan the viscosity falls to just over 0.05Pa.s and is not affected by time of measurement. This indicates a compatible biopolymer solution. These results show that the replacement of LBG by λ-carrageenan causes a recovery in viscosity values. At 20:80 and 40:60 λ-carr:LBG there is an increase in the viscosity drop as time progresses. This is possibly due to a delaying of the phase separation process. The inclusion of λ-carrageenan does alter the proportion of LBG and since a reduction in LBG concentration will improve its miscibility with gelatin this will change the phase behaviour. The control of 0.5% λ-carrageenan in the absence of LBG and gelatin shows a constant viscosity value of around 0.028Pa.s. This is lower than for the LBG-containing sample and is further evidence of the compatibility between gelatin and λ-carrageenan.

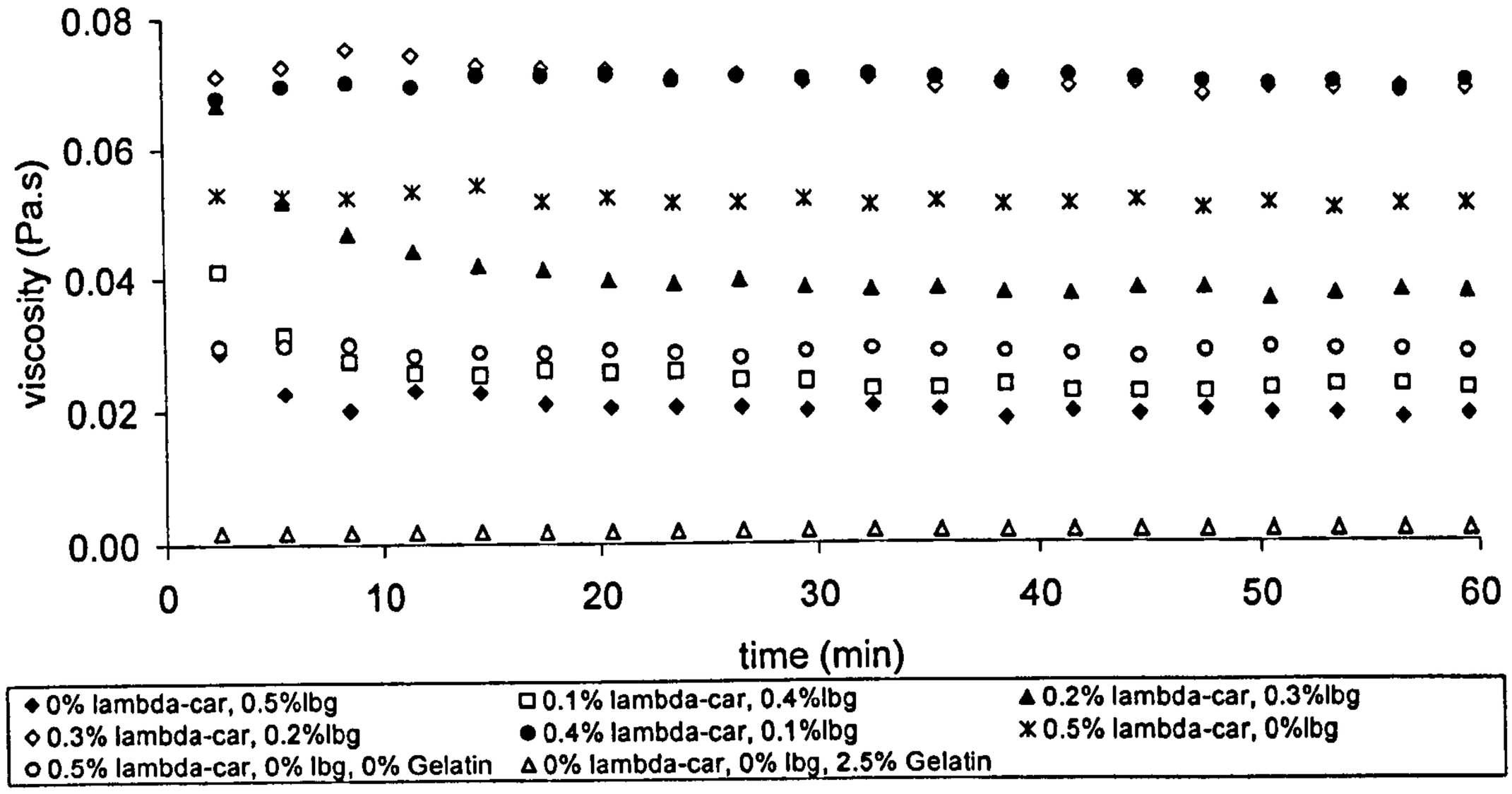


Figure 4-12 Change over 60 minutes in the flow behaviour of mixtures of 2.5% gelatin plus varying ratios of lambda carrageenan and LBG. Measured at 40°C using 4°/40mm cone and plate geometry. Shear rate $1s^{-1}$.

Work by Alves, Garnier et al. 2001 has shown concentration dependent phase composition for mixtures of 0.8% w/w LBG with type B gelatin. At μ=0.002M and pH 5.0, just below the isoelectric point, gelatin continuous mixtures were observed above 2.7% gelatin. In the mixture used in this study the μ=0.116M and the pH of 6.8 was well above isoelectric point of 5, the gelatin therefore had a net negative charge. The deviation from the isoelectric point greatly reduces the tendency for gelatin to come out of solution.

4.4. Phase composition analysis

In order to determine with more certainty the composition of the separated phases of the 0.5% Sigma LBG/2.5% Croda gelatin mixtures, compositional analysis was undertaken using Lowry protein determination for the gelatin and total sugar for the LBG. Samples were prepared by taking mixtures of locust bean gum and gelatin solutions and combining these at 80°C for 30 minutes, stirring continuously. The samples were left to stand for 2 hours at 50°C before centrifuging at 600rpm for 20 minutes to form two distinct phases. The phases were collected for further analysis. The supernatant had a transparent appearance, resembling a pure gelatin solution, whereas the precipitate was opaque.

4.4.1. Cooling run in oscillation

The collected supernatant and precipitate were subjected to oscillation during cooling from 40 to 15°C. This revealed the supernatant to have a G'/G" crossover of 17.8°C and a G' at 15°C of 52Pa. The precipitate had a G'/G" crossover of 17.7°C and a G' at 15°C of 226Pa. The gelation temperatures were similar to that observed for a 2.5% gelatin solution. The G' value at 15°C for the supernatant is also that which is expected for 2.5% gelatin. The G' value at 15°C for the precipitate is much higher than expected; indeed a comparison of the raw data showed it to be dissimilar to that of a pure gelatin solution. These findings confirm that both phases contain gelatin, which is able to form a continuous network.

4.4.2. Protein determination of separated phases

The recovered phases were subjected to a mass balance and protein determination using the Sigma Lowry protein determination method. Samples were dried overnight at 105°C. A standard curve was produced using BSA protein standard and the absorbance of the standards and diluted samples were read at 750nm.

The drying data showed a division of dried material between the two phases to be 1.84% material in the wet supernatant and 0.87% in the wet precipitate. Correcting for moisture at 12.6% for the gelatin and 10% for the LBG, the combined recovered total was exactly 3%. This was the same value as the non-moisture adjusted powders actually used to prepare the solution.

Using the above data it was possible to calculate the protein content of each phase from the standard curve. The protein amount estimated from the standard curve was 233.0mg for the supernatant and 124.4mg for the precipitate. Correcting for dilution, these represented actual protein per gram of starting material of 0.932 for the supernatant and 0.498 for the precipitate. The percentage of protein was therefore estimated to be 1.94% for the wet supernatant and 0.49% for the wet precipitate, a total of 2.43% protein. This represents a slight underestimation of gelatin in the samples.

4.4.3. Determination of galactomannan by total sugar analysis

	Supernatant (mg/g)	Precipitate (mg/g)
Mannose	$1.72*10^{1}$	$2.53*10^{2}$
Galactose	$7.53*10^{0}$	$7.27*10^{1}$
Glucose	$3.03*10^{0}$	0
Total	$2.47*10^{1}$	$3.25*10^{2}$

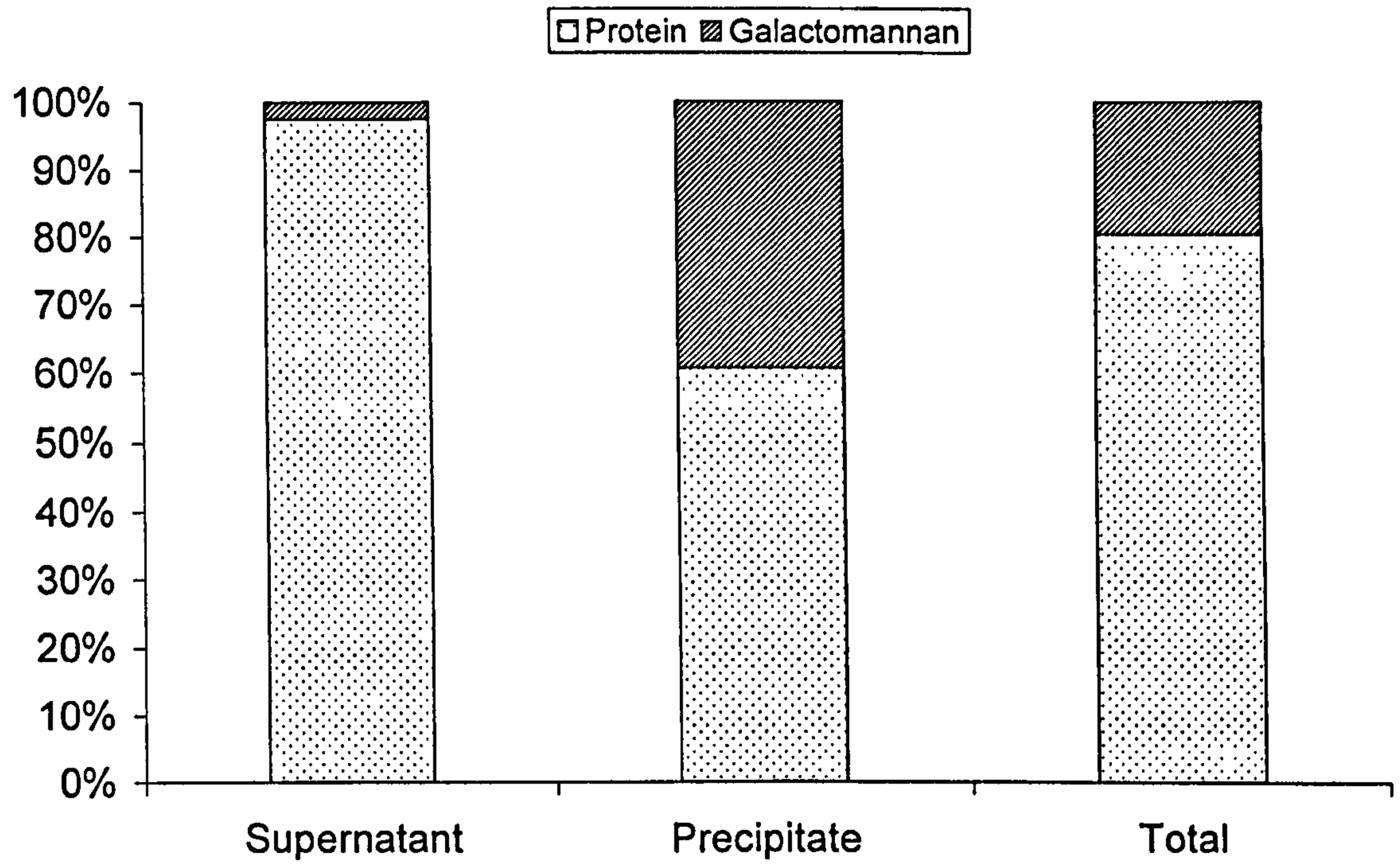
Table 4-1 Sugars (mg/g) of dried supernatant and precipitate from phase separated Sigma locust bean gum/Croda gelatin mixture.

Table 4-1 shows mannose and galactose amounts measured by total sugar analysis. The ratio of mannose to galactose for the supernatant and precipitate is 1.9 and 3.5 respectively. The ratio for the precipitate is in accord with the value expected for LBG (Daas, Schols et al. 2000). This indicates that the galactomannan present in the supernatant has a higher degree of substitution, with a ratio that is in fact closer to guar than LBG. It is known that guar is readily cold-water soluble, unlike LBG (Picout, Ross-Murphy et al. 2002). The ratio reported here may, therefore, be due to partitioning of the high and low solubility fractions of the LBG. The presence of glucose in the supernatant has also been reported to occur in LBG samples (Kok, Hill et al. 1999).

The respective proportions of sugars within the phases enable the composition of the phases to be estimated. Using the moisture-adjusted drying values from the protein determination, 312mg sugars per 100g wet precipitate and 50mg per 100g wet supernatant were estimated. Expressed as a percentage the measured sugars accounted for 32.53% and 2.47% of the total dried mass respectively. Crucially, this shows that the composition of the supernatant is almost exclusively non-galactomannan.

A protein and galactomannan composition can be conducted for the two phases to reveal which phase is enriched with which biopolymers. Figure 4-13 shows the combined analysis for each phase. Whilst total recovery of material was not achieved it is obvious the supernatant is predominantly protein, whereas the precipitate contains more even proportions of each component.

It is generally accepted that mixtures of a neutral and a charged biopolymer tend to have improved miscibility (Piculell, Bergfeldt et al. 1995). However, LBG and type B gelatin have been shown to exhibit incompatibility over a wide range of pH, ionic strength and temperature (Alves, Antonov et al. 1999).



Phase	Protein (mg/g dry weight)	Galactomannan (mg/g dry weight)	Total
Supernatant	931.8	24.7	956.5
Precipitate	497.6	325.7	823.3

Figure 4-13 Comparison of amounts of protein and galctomannan in each phase.

4.5. Conclusion

The purpose of the work reported here was to show whether phase separation occurred in ternary mixed gels of the bioplymers studied, at which concentrations this phase separation occurred, and to show the nature of the phases present using rheological and compositional analysis. Viscosity studies excluding the gelling kappa carrageenan were undertaken to provide supporting data.

Turbidimetric studies at 490nm showed clearly that phase separation, attributable to the biopolymers, occurred in mixed gels of k-carrageenan, gelatin and LBG, depending on gelatin concentration. Visual comparison revealed large phase separated inclusions present within a clear continuous phase. These showed that concentrations of gelatin at 2.5% and above were sufficient to cause macroscopic phase separation.

Having established that phase separation was present, a closer investigation of the concentration of gelatin required to cause a rheological change in gelled mixtures was undertaken. Small deformation studies revealed that gelatin causes gel weakening at all concentrations at 25°C after cooling. Phase inversion based on melting point change was not observed in these mixtures at any gelatin concentration, although recovery of elastic modulus was observed at 10% gelatin.

Large deformation studies conducted using the TA-XT2i equipment revealed a more complex situation that required investigation of the curing variables of time and temperature. A model was created using experimental design software, which revealed a highly negative impact on gel breakstrength at concentrations in excess of 2.5% gelatin. This finding was significant when related to data in table 3-1, which showed that gelatin concentrations in product ranged between 1.2 and 2.8%. Viscosity studies using lambda carrageenan showed the improved miscibility of mixtures when a charged polysaccharide is present. This helps us understand better the situation in the gelled mixtures.

In order to probe the likely composition of the continuous phase, rheological measurements of LBG/gelatin mixtures revealed gelatin-melting temperatures for

both supernatant and precipitate. A reduction in modulus was observed for the precipitate consistent with the presence of phase separated LBG inclusions. Visual observations confirmed this. Protein and sugars analysis confirmed the composition of the supernatant to be almost exclusively gelatin, with possibly some high solubility galactomannan present. The precipitate contained a significant proportion of gelatin, but had a greater amount of LBG than the supernatant.

5. The protective role of gelatin during thermal processing

5.1. Introduction

It has been observed that autoclaved mixtures of LBG and κ–carrageenan mixtures have greater structural integrity when heated in the presence of the meat constituents, compared with systems containing just the polysaccharides and salts.

A possible reason for this is that gelatin released from the meat constituents on autoclaving helps maintain overall gel strength. Mechanisms for this could be:

(1) The formation of a gelatin gel on cooling makes an additional contribution to the total gel strength.

(2) That the gelatin and the mixed locust bean gum/carrageenan system phase separate. The concentration of the latter is thus enhanced in its own domain. If this is the continuous phase the gel strength of the total system can be enhanced.

(3) Gelatin protects the polysaccharides from degradation.

The kinetics of depolymerisation are dependent on the biopolymer type, temperature and solution properties such as pH and ionic strength. It is accepted that where pH is in the neutral range, biopolymer degradation is generally caused by oxidative/reductive depolymerisation (ORD) (Wellington 1983). Antioxidant strategies have proved to be effective at reducing degradation due to ORD during thermal processing (Hill and Gray 1999).

The objective of this work was firstly to confirm the enhanced gel strength after autoclaving for carrageenan/locust bean gum in the presence of gelatin, secondly to determine what mechanism was responsible for the gel weakening, and lastly to decide between the three possible suggested causes of this effect.

Large deformation studies and the creation of an experimental model on the effect of gelatin on carrageenan/LBG mixtures were combined with viscosity studies to reveal the effectiveness of gelatin in protecting polysaccharides through the autoclaving

cycle. Part of the viscosity work included the use of protease and beta-mannanase enzymes, to determine the contribution of the individual biopolymers to viscosity. Viscosity provided an indirect indication of molecular weight. Direct estimation of molecular weight was achieved using light scattering. An attempt was made to build a full picture of behaviour in the experimental systems, including the important role of buffer ionic strength in determining the gel strength of the autoclaved biopolymer mixtures.

5.2. The effect of gelatin on the large deformation behaviour of autoclaved gel mixtures

5.2.1. Initial observation

One of the key drivers for the project was the reported difference in gel strength between autoclaved polysaccharide blends and the real product, as observed by the sponsoring company. This meant that it was not possible to predict gel strength in the product on the basis of the quality tests on the gel strength of the polysaccharides. The objective of the work described in this section is to determine if released gelatin could be the cause of the observed differences in the gel properties. Because of the need to show relevance to the real system, the buffer employed in petfood manufacture was used for this work (TKPP). This buffer had a pH adjusted to 7.0. It was found that the pH fell to 6.8 with the biopolymers added and reduced to 6.7 after autoclaving. The ionic strength was calculated as μ=0.56 for the TKPP buffer. This compares with 0.1 for the buffer system used in this section.

Figure 5-1 shows breakstrength data for mixtures subjected to mild and severe heating at 0 and 5% gelatin. Autoclaving gelatin-free gels has a large effect resulting in a >85% reduction in breakstrength. At 5% gelatin there is a slight increase in breakstrength for the mild heat treatment, but after severe heating the breakstrength retains over 70% of its pre-autoclaved value. These results show that gelatin has a beneficial effect on breakstrength of carrageenan/LBG mixtures after autoclaving.

The reader should note that these experiments were conducted on samples prior to the centrifugation purification step employed for the LBG and can not be directly compared with subsequent breakstrength data.

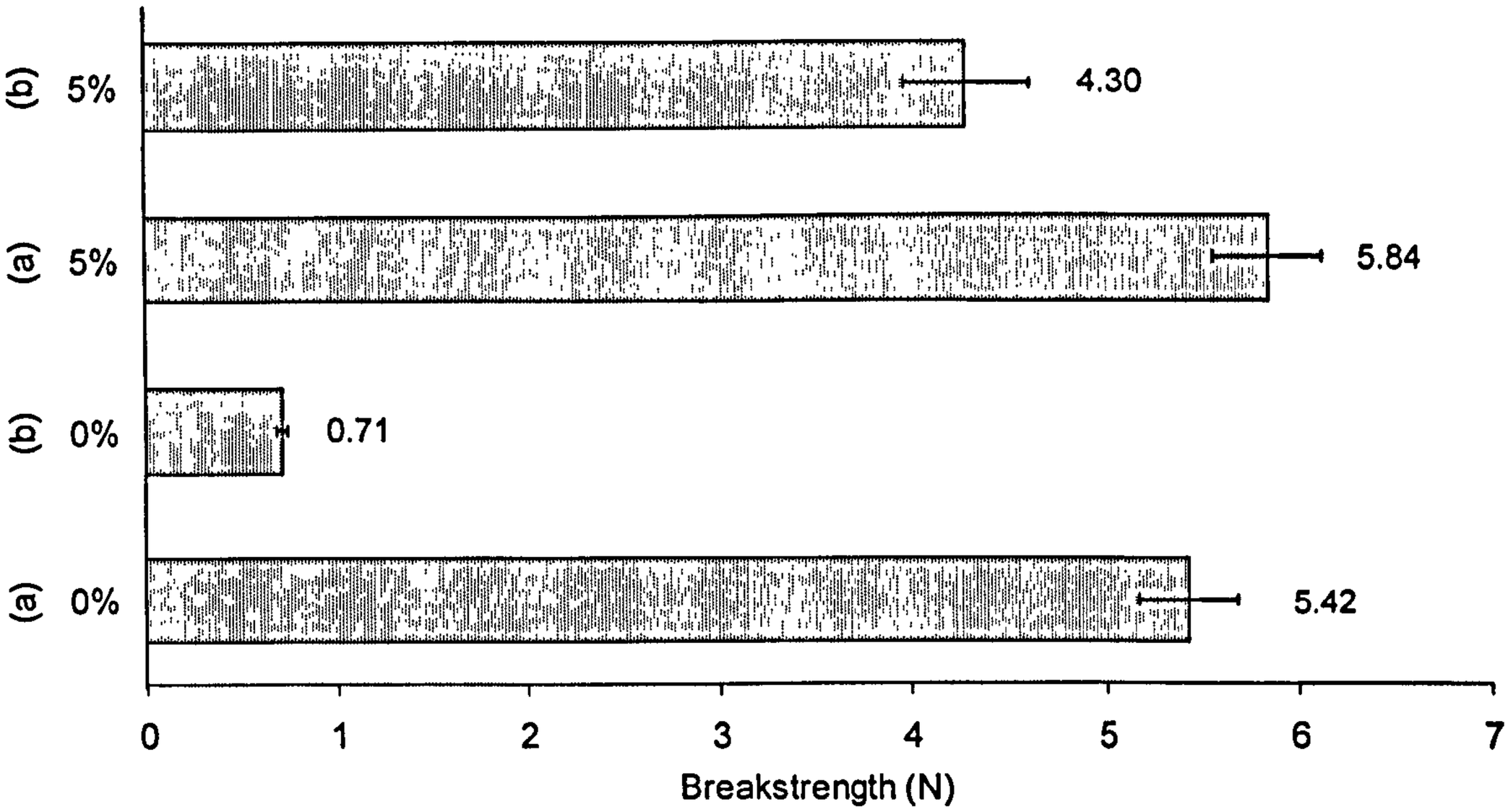


Figure 5-1 Breakstrength of 0.3% cLBG and 0.3% carrageenan at 0 and 5% Sigma gelatin and after heating at 80°C for 30 minutes (a) and 121°C for 50 minutes (b). Buffer used was TKPP and gels were cured overnight at 5°C. Error SD of 5 repetitions.

5.2.2. Model of the effect of gelatin concentration and autoclave time on mixture breakstrength

In order to gain a fuller understanding of gel behaviour, a model was constructed relating gelatin concentration and autoclave time to gel breakstrength after curing under controlled conditions. Experimental conditions were between the limits of 10 and 50 minutes autoclaving and 1 and 5% gelatin concentration. The experimental data was fitted to a model containing linear, squared and first order interaction terms. Gel strength was then related to gelatin concentration, c(%), and autoclave time, t(min), by:

$$Breakstrength(N) = 4.54\times10^{0} - 7.30\times10^{-1}\times c - 1.53\times10^{-2}\times t + 1.79\times10^{-1}\times c^{2} - 2.05\times10^{-3}\times t^{2} + 4.76\times10^{-2}\times c\times t$$

Equation 5-1 Model of polynomial relationship between gelatin concentration, autoclave time at 121°C and breakstrength. Numbers shown are actual factors.

The fit of the data to the model and all model terms had a significance of >99.99%, which means there was <0.01% chance of the model being generated by chance. The model adjusted r-squared, 0.9753, and the predicted r-squared, 0.9541, which are measures of the amount of variation, in the first case, around the mean explained by the model and, in the last case, in predicted data explained by the model. The closeness of these values to each other also shows model reliability. A response surface plot of the model data is given in figure 5-2. This shows the regions where gel strength is highest to exist at the bottom left and top right of the plot i.e. showing that gelatin has a negative effect at around 10 minutes autoclave time and a positive effect at the highest times.

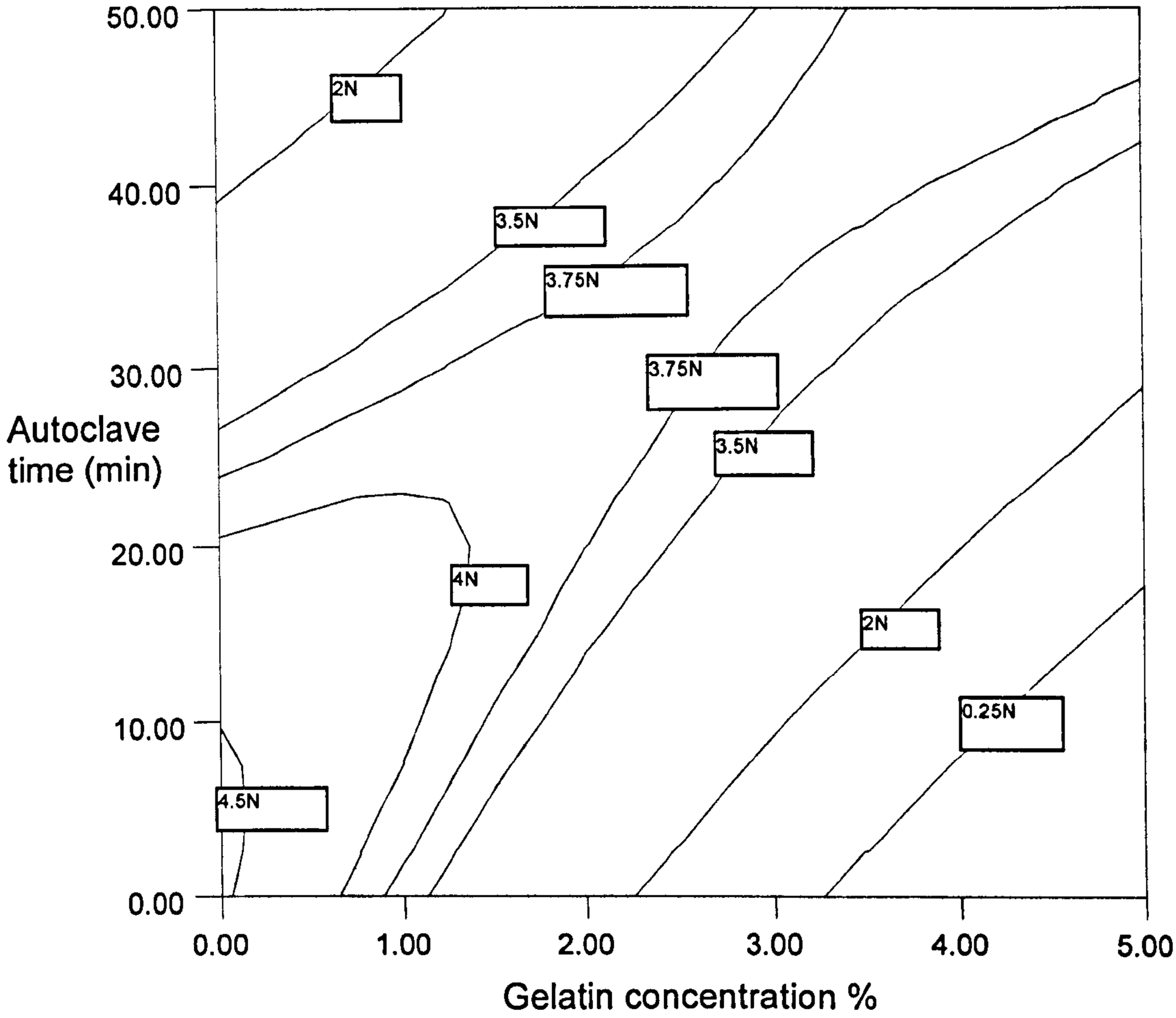


Figure 5-2 Response surface of predicted breakstrength showing the effect of gelatin concentration and autoclave time. Carrageenan/LBG at 0.6% gels were cured for 1h at 5°C and made using TKPP buffer.

The main features of interest from this model are illustrated in figures 5-3 and 5-4. Figure 5-3 shows the predicted effect of gelatin concentration on the breakstrength when the system is autoclaved for 50 minutes. Gel strength increased with gelatin concentration up to a plateau value at about 4% gelatin.Figure 5-4 compares the effect of autoclaving on the gel breakstrength of the carrageenan/LBG system in the presence and absence of 2.5% gelatin. In the absence of gelatin breakstrength decreases continuously with autoclaving time. The inclusion of gelatin provides substantial protection to gel strength loss at the higher autoclaving times. In the presence of 2.5% gelatin the breakstrength is almost independent of autoclaving time between 20 and 50 minutes.

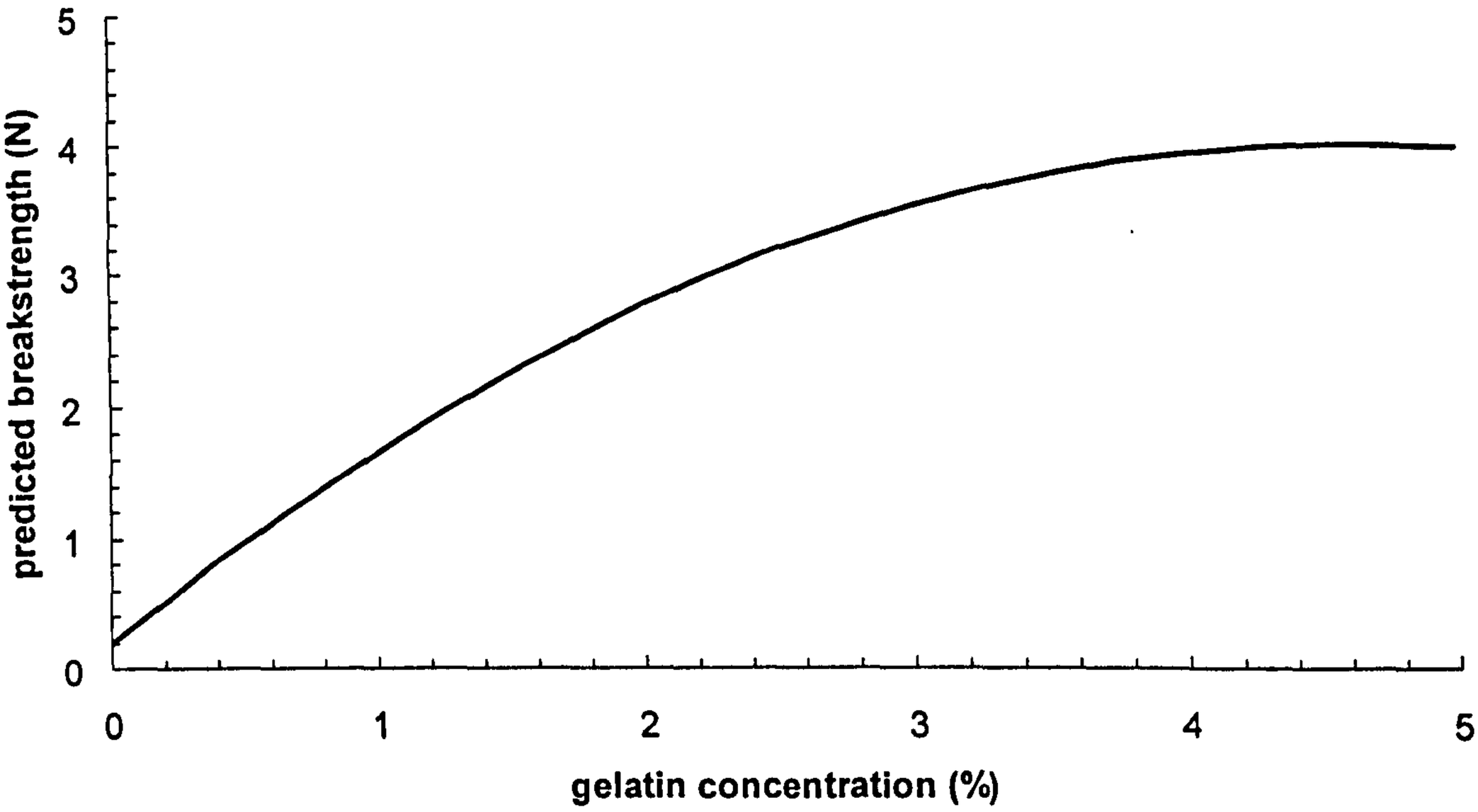


Figure 5-3 Predicted breakstrength values for 0.3% carrageenan/ 0.3% LBG gels autoclaved for 50 minutes with increasing limed ossein gelatin concentration.

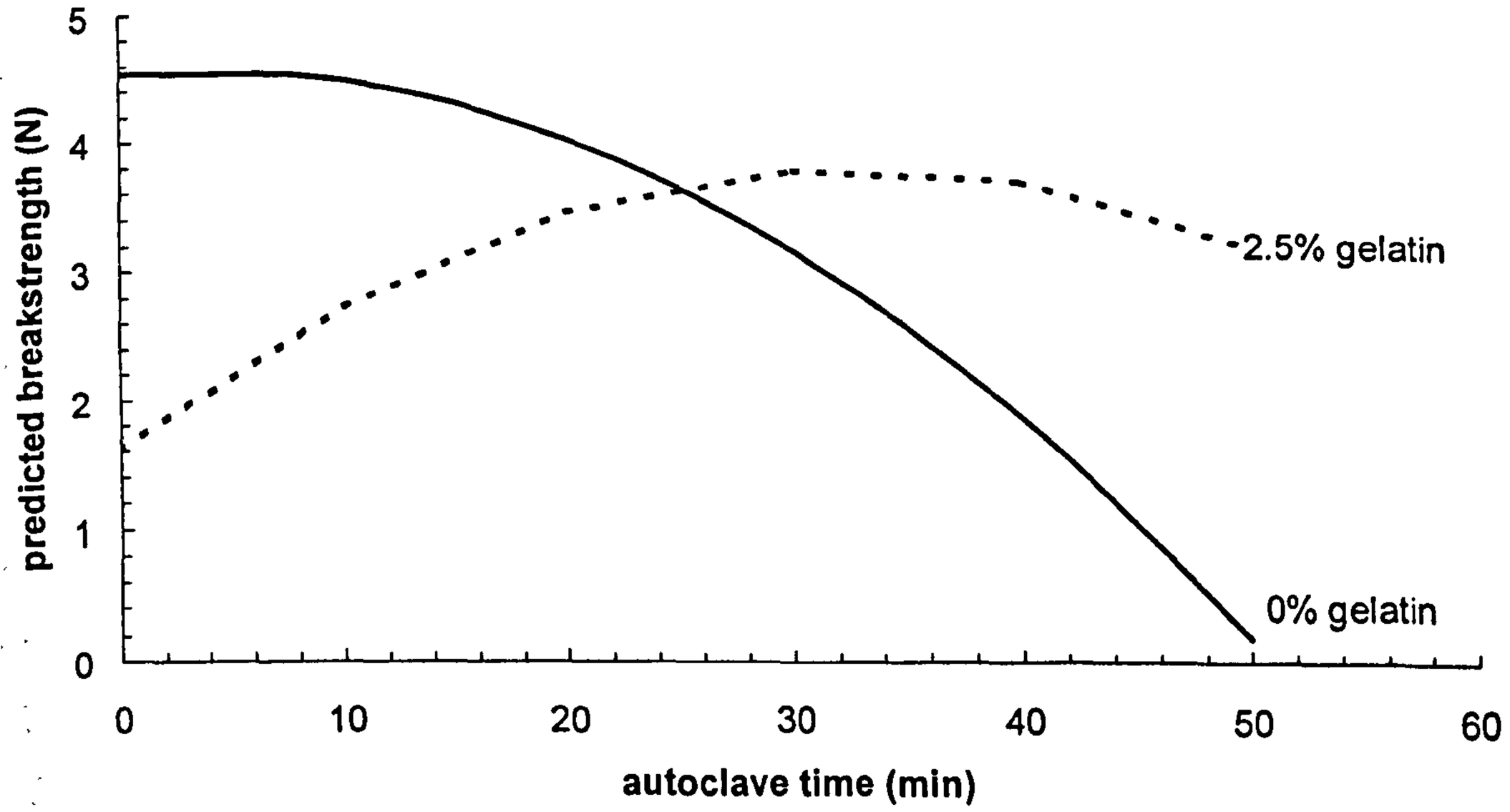


Figure 5-4 Effect of autoclave time on breakstrength for 0.3% carrageenan/ 0.3% LBG gels in the presence and absence of gelatin.

5.2.3. Effect of buffer type on breakstrength after autoclaving

There was a need to compare buffer type in order to rationalise viscosity data obtained using the pH 6.8, μ=0.1 buffer with the data incorporated in the model, which had used the TKPP buffer, pH 7.0, μ=0.56. Figure 5-5 shows a comparison of gel breakstrength measurements using both buffers for mixtures containing 0 and 2.5% gelatin and for both heat treatments. This data shows that the TKPP buffer yielded the result predicted by the model and the result shown by figure 5-1. The data reveals a spectacular collapse (>80% loss) in breakstrength after autoclaving. At 2.5% gelatin there is a reduction in breakstrength pre-autoclaving, which is attributed to phase separation. Post-autoclave, the breakstrength accounts for 85% of the non-autoclaved, gelatin free mixture. The phosphate pH 6.8 buffer yielded unexpected results. Apart from a very similar value for the gelatin free, non-autoclaved sample, the rest of the treatments had different behaviour from the samples containing the TKPP buffer. After autoclaving, the gelatin free sample had a higher breakstrength of 1.6 compared to 0.5N with TKPP. The reduction in breakstrength of the non-autoclaved sample with gelatin was much greater than with TKPP at close to 85%. Most interesting were the very similar values returned for both the autoclaved samples, regardless of gelatin concentration at 1.7 with 2.5% gelatin and 1.6N without.

These results seem to confirm the idea that thermal degradation can increase mixture breakstrength by improving mixture compatibility. The difference in behaviour between what is essentially a high ionic strength buffer and the lesser phosphate pH6.8 buffer shows the impact that salt plays in affecting phase behaviour. The TKPP buffer has both the most damaging effect on the gelatin free mixture and the greatest protective effect of the gelatin on autoclaving. The question is, what interaction occurs between the TKPP buffer and gelatin to exhibit the protective effect seen in these mixtures? High ionic strengths promote compatibility in associating gelatin/carrageenan mixtures (Michon, Cuvelier et al. 1995), (Antonov and Goncalves 1999). Antonov observed that association was still possible even when μ=0.35. Above μ=0.8 Michon observed turbidity in gels of 3% acid gelatin and 0.9% iota-carrageenan. In our system, the impact of high ionic strength, μ=0.564, will tend to promote segregation of carrageenan from gelatin, although it is likely the carrageenan

concentration may well be below the threshold for separation. In any case the driving force promoting phase separation is somewhat negated at autoclave temperatures. It seems more likely that high ionic strength enhances the protective effect of gelatin in these systems. A greater μ may cause flocculation of non-gelatin, LBG protein, which reduces its capacity to protect. This would allow the gelatin protective effect to be more pronounced and would explain where the naturally occurring effect is present i.e. in the μ=0.1 buffer gelatin appears ineffective.

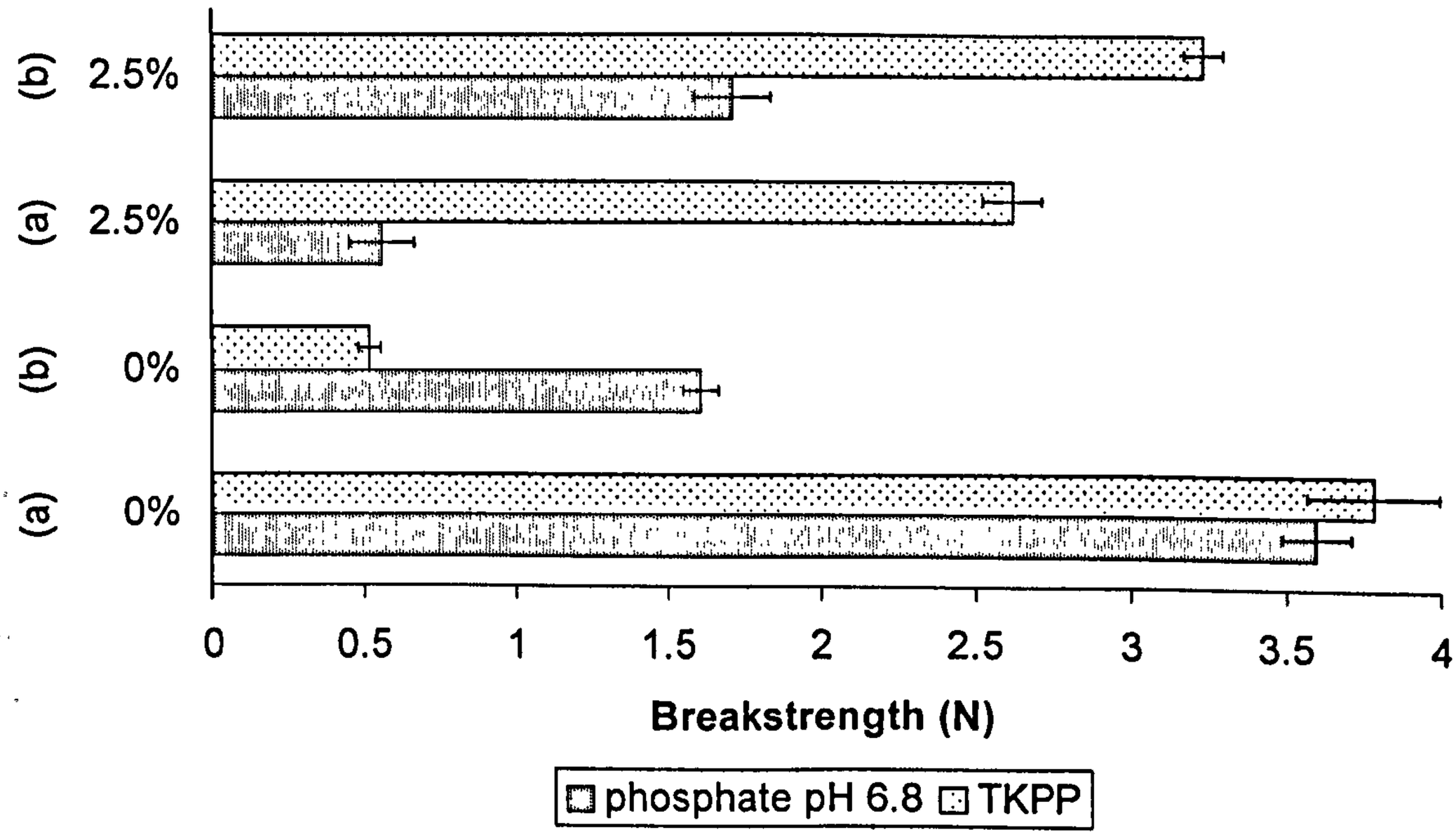


Figure 5-5 Comparison of effect of buffer type on breakstrength 0.3% carrageenan, 0.3% LBG mixtures with and without gelatin. Gels were cured for 1 hour at 5°C. (a) heated at 80°C for 30 minute, (b) heated at 121°C for 60 minutes.

5.3. Molecular Weight Effects

5.3.1. The effect of bloom value of gelatin on mixture breakstrength

Concentration and molecular size are key variables in determining phase separation in mixtures of incompatible biopolymers. The threshold concentration, below which mixture compatibility is achieved, is an important concept and exists due to a favourable entropy of mixing dominating the unfavourable interaction enthalpy. This driving entropic force becomes satisfied at relatively low concentrations, after which the enthalpic interactions dominate. It is possible to increase or decrease the threshold concentration for separation by changing the molecular weight of the biopolymer concerned, in this case gelatin. Reducing the molecular weight increases the threshold concentration for separation.

A study was conducted to reveal the effect of gelatin molecular weight on the mixture breakstrength. We have already described the effect of gelatin on non-autoclaved gels in the previous chapter, showing significant gel weakening at commercially important concentrations. In a sense this work formed a bridge between two of the main ideas behind this project, which are phase separation and degradation behaviour. Figure 5-6 shows the significance of gelatin bloom value on gel breakstrength in these mixtures. At a concentration known to cause gel weakening it is possible to generate gel strength recovery by using a mixture of a lower bloom value. Croda 305, which has the greater molecular weight, clearly has a reducing effect compared to Croda 106 with values of 2.01 and 4.24N respectively at 30 minutes. There is little change after a further 30 minutes suggesting the structure had stabilised.

What this finding shows is that there is an inverse relationship between mixture gel strength and the bloom strength of the gelatin. This could mean that a molecular weight lowering process, such as autoclaving, could increase mixture breakstrength.

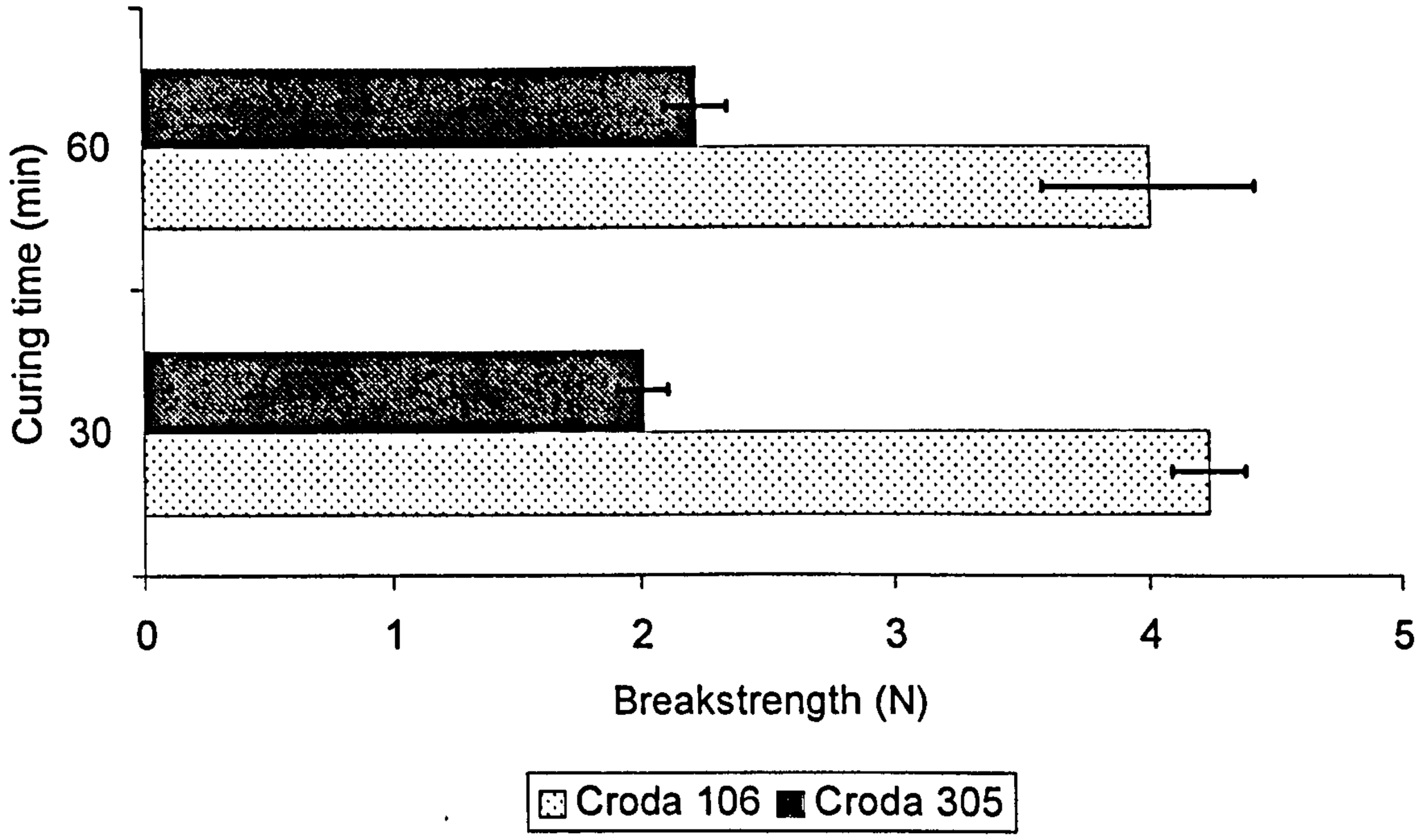


Figure 5-6 Breakstrength of non-autoclaved mixtures with different bloom gleatins: Croda 106 and Croda 305 at 2.5% concentration. Cure temperature 5°C. Carrageenan/LBG at 0.6%. Error as standard deviation.

5.3.2. Molecular weight changes of autoclaved carrageenan/LBG mixtures in the presence of gelatin.

At the beginning of the chapter, three possible interpretations were discussed. The model and the data in Chapter 4 clearly show that for non-autoclaved systems, gelatin addition reduces gel strength. This reduction is a consequence of phase separation. It is therefore unlikely that either phase separation or an increase in gel strength due to the contribution of a gelatin gel can be the reason for the enhanced gel strength in the autoclaved systems. The remainder of this chapter will therefore concentrate on the hypothesis that gelatin sustains mixture gel strength by protecting the polysaccharides from degradation.

The principal changes to the experimental mixture were concerned with the impact on polysaccharide molecular weight when heated to autoclave temperature in the presence of gelatin. Direct measurement of molecular weight in a gelled mixture of three biopolymers was not possible, so an alternative was necessary.

Viscosity is related to molecular weight on the basis of the Mark-Houwink Equation:

$$[\eta] = K_M [M_W]^{\alpha} \qquad \text{Equation 5-2}$$

where [η] is intrinsic viscosity, M_w is molecular weight, K_M (Mark-Houwink constant) and α are constants. The intrinsic viscosity is obtained by an extrapolation to zero concentration. An approximation for LBG in the concentrated domain (C>C*) used by Kok from the work of Bradley and Mitchell, 1988, related measured viscosity to molecular weight as follows:

$$\eta \sim \propto (M_w)^3 \qquad \text{Equation 5-3}$$

5.3.2.1. Viscosities of individual and mixed solutions

The objective of these experiments was to determine to what extent degradation of polysaccharides could be reduced by protein addition. As has been stated viscometry was selected as a method that could be used to give information on changes to molecular weight. In this study viscosity was used to show the effect of autoclaving on separate solutions of gelatin and carrageenan/LBG compared to a combined mixture. For practical reasons and for consistency all samples were diluted 1:1 prior to measurement. The more conventional pH 6.8, μ=0.1, buffer was used in these studies. Figure 5-7 shows the viscosity change with increasing heating severity of Croda 305 gelatin, a mixture of carrageenan/LBG and the same mixture in the presence of the gelatin. Pre-autoclaved viscosities were 5.75 and 6.95mPa.s for the polysaccharides alone and the mixture respectively. The response of the mixture with progressively longer autoclaved times shows a greater resistance to viscosity loss than with the polysaccharides alone. The viscosity loss appears to follow a linear trend from 5 minutes onwards in both cases. After 60 minutes the viscosity fell to 3.04mPa.s for the polysaccharides alone, and 4.65mPa.s for the mixture. This represents a fall of 47 and 33% respectively. Excluding the value for 0 minutes autoclaving time it is possible to gain a linear fit of the subsequent data with respective r-squared values of 0.963 and 0.997. These results show that adding gelatin

has some beneficial effect on the viscosity after autoclaving, which indicates some element of difference from the breakstrength data gained using the pH 6.8, μ=0.1 buffer.

Also, figure 5-7 shows a marked reduction in the viscosity of gelatin alone between 0 and 15 minutes autoclaving time. The residual viscosity after 5 minutes is 34% of the 6.97mPa.s starting value, no further reduction is observed after 15 minutes. This residual value compares with 80% of the starting value for the polysaccharides at the same time interval and indicates the relative ease of degradation for gelatin at the temperature of processing.

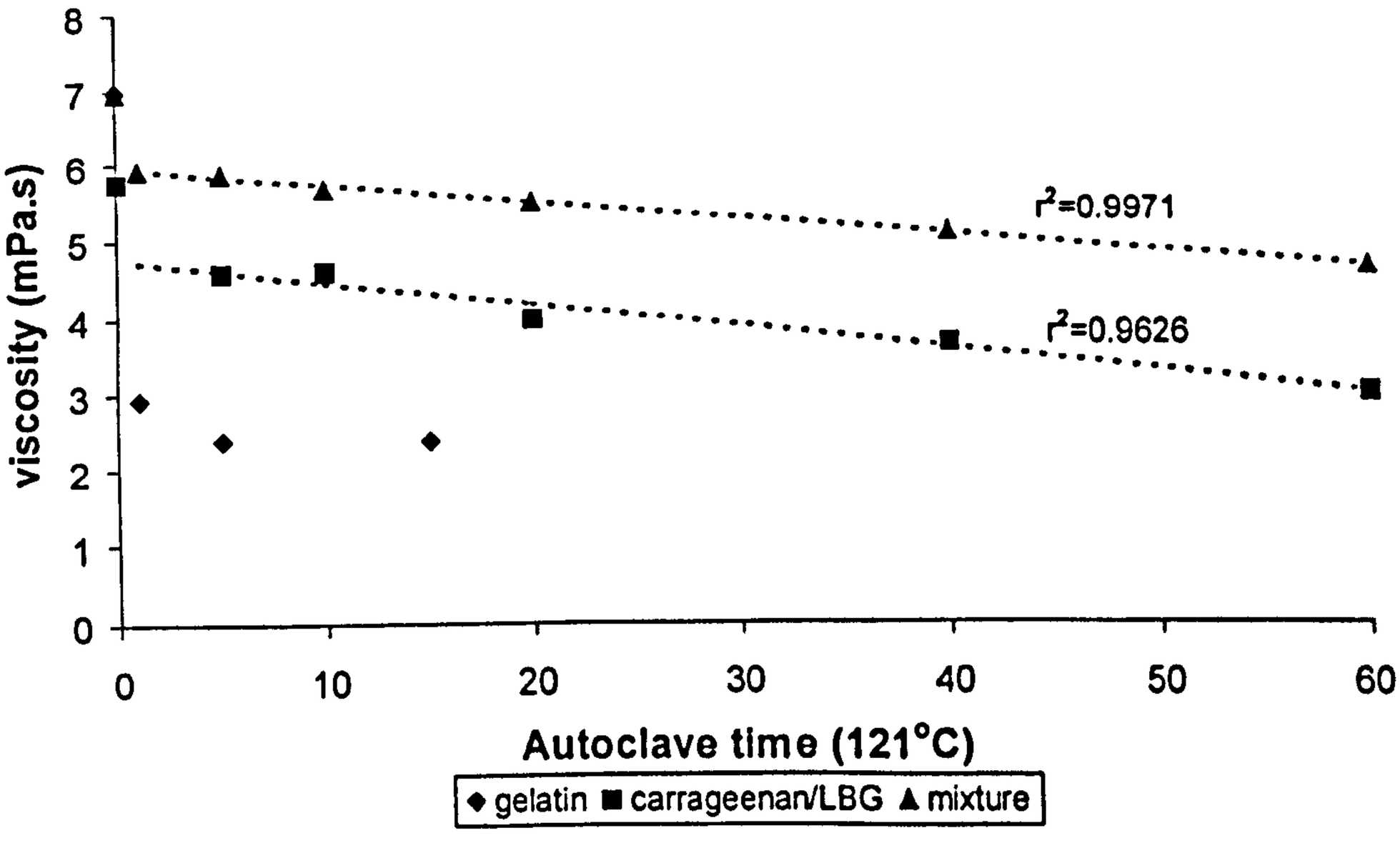


Figure 5-7 The effect of increasing severity of heating process on viscosity for separate and combined mixture components.

5.3.2.2. Concentration effect on LBG degradation

To determine if locust bean gum is able to confer a level of self-protection, the viscosity response of LBG solutions autoclaved for 60 minutes at different concentrations, but measured at 1% at 40°C, was determined. The results are shown in figure 5-8. Up to a heated concentration of 3% there is a steady increase in viscosity. Above 3% concentration there is no increase in viscosity found at 4 or 5%. A probable explanation is that the increase in concentration may cause a change from convection to a conduction pack, resulting in a lower rate of heat transfer. Thus at 3% and above, heating is by conduction and the severity of heat treatment in this range will be independent of concentration. It is also possible that LBG self protects as suggested above. At lower concentrations, water is in excess up to a critical concentration. Below this critical concentration, extra LBG lowers molecular mobility, which gives greater protection as the dissolution of the LBG is reduced. Above the critical concentration, no further reduction in mobility is possible as the water is completely bound to the LBG and not free to aid molecular motion.

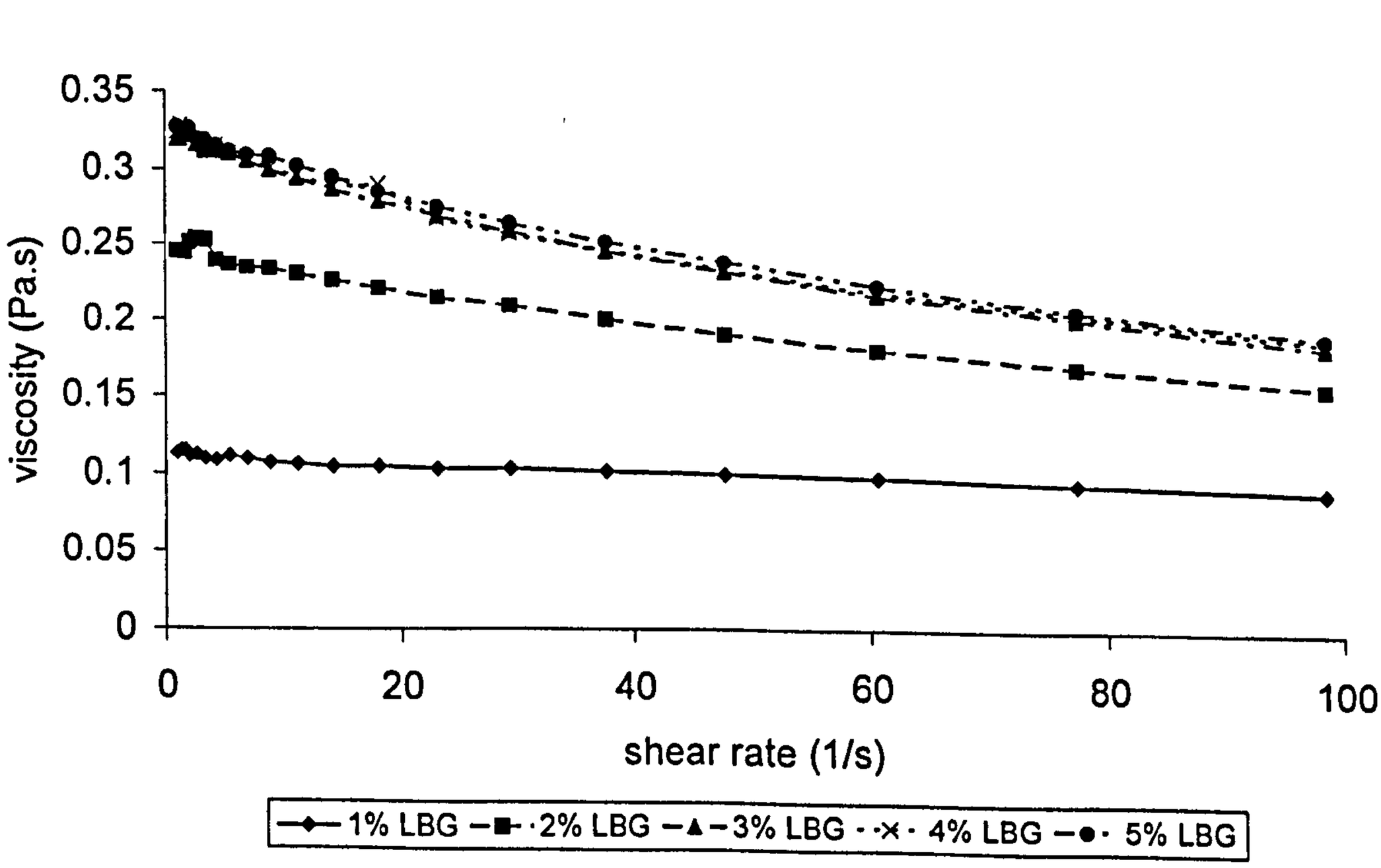


Figure 5-8 Viscosity response of LBG solutions autoclaved for 60 minutes at the concentrations shown. Measured at 1% concentration after dilution.

5.3.3. Determination of effect of thermal processing on biopolymers using enzyme digestion

The work carried out in this section uses specific viscosities, which are described below:

$$\eta_{sp} = \frac{\eta}{\eta_{sol}} - 1 \qquad \text{Equation 5-4}$$

Where η_{sp} is the specific viscosity, η is the apparent viscosity in Pa.s and η_{sol} is the solvent viscosity in Pa.s.

Figure 5-9 shows the effect of the enzyme treatment on the specific viscosity. The beta-mannanase produced the greatest overall viscosity reduction for autoclaved and non-autoclaved samples in the presence and absence of gelatin. The addition of gelatin, across the concentration range, to the mixtures before autoclaving had no effect on the specific viscosity values, the values being approximately 6.5 before and 1.2 after mannanase/protease digestion at $6s^{-1}$. After autoclaving there was an increase in mixture-specific viscosity with increasing gelatin in the pre-digested mixtures from 2.94 at 0% to 5.69 at 2.5% gelatin. The specific viscosity value for the mixtures after autoclaving and also with the enzyme treatments to degrade the LBG and gelatin was around 1. This shows that gelatin has a minimal effect on the viscosity of the pre-autoclaved mixtures and the enzyme treated samples. In addition, figure 5-10 shows the viscosity values for the mixtures after the mannanase digestion, the viscosity value representing that of the gelatin and the carrageenan less the LBG. The values for both sets of samples are similar, around 1.2 for the autoclaved mixtures and 1.6 for the non-autoclaved mixtures across the gelatin concentrations. This shows that gelatin makes little contribution to viscosity and its contribution after autoclaving is even smaller. It would therefore appear that it is changes to the LBG that contribute to the marked changes in viscosity and breakstrength of the mixed system.

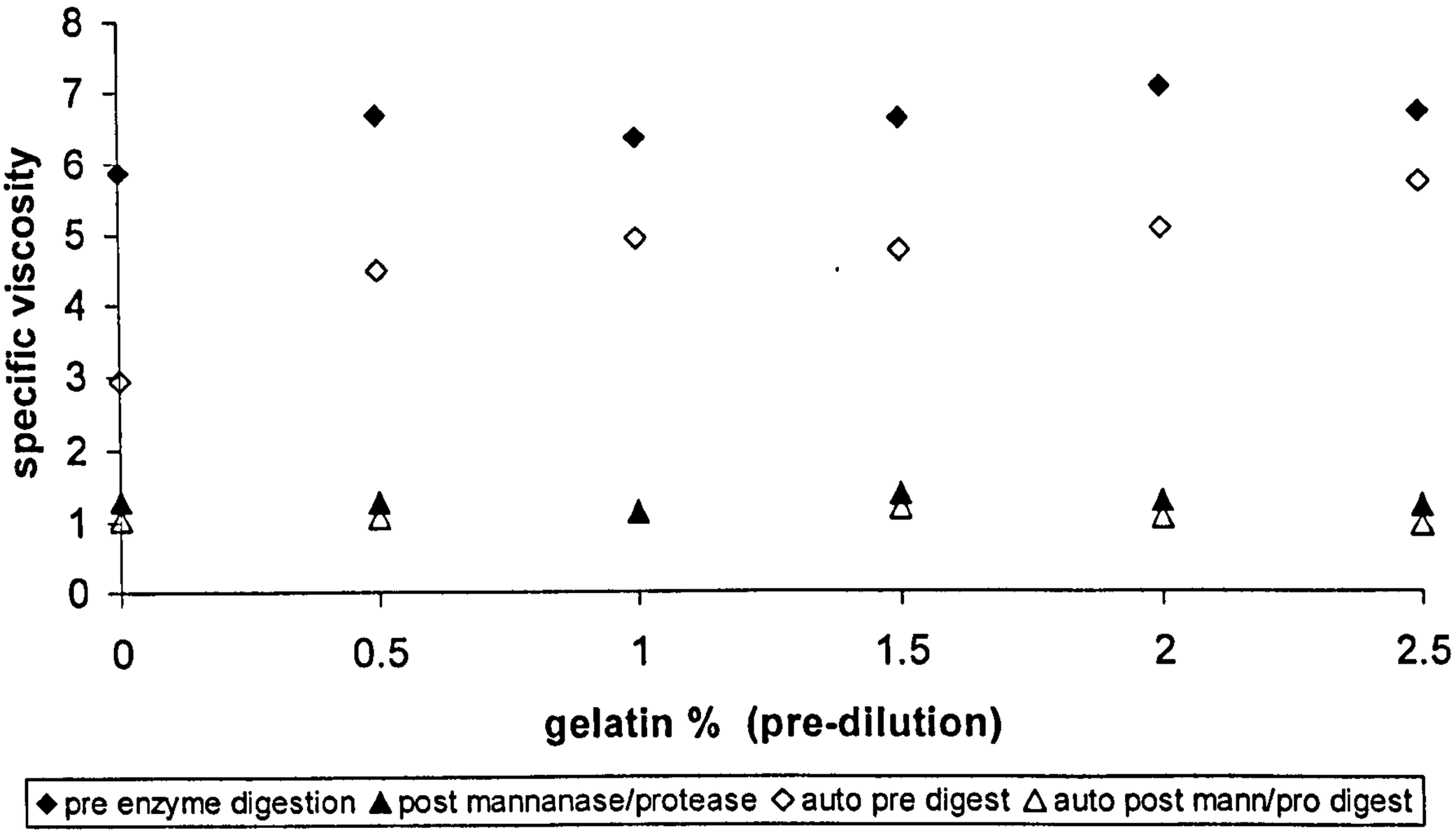


Figure 5-9 The effect of limed ossein gelatin on the viscosities of autoclaved (auto) and non-autoclaved carrageenan/ locust bean gum mixtures, before (pre) and after (post) enzyme treatment with beta-mannanase and protease. Viscosity measured at $6s^{-1}$ and at $50^{o}C$ using double gap geometry after 1:1 dilution in sample buffer.

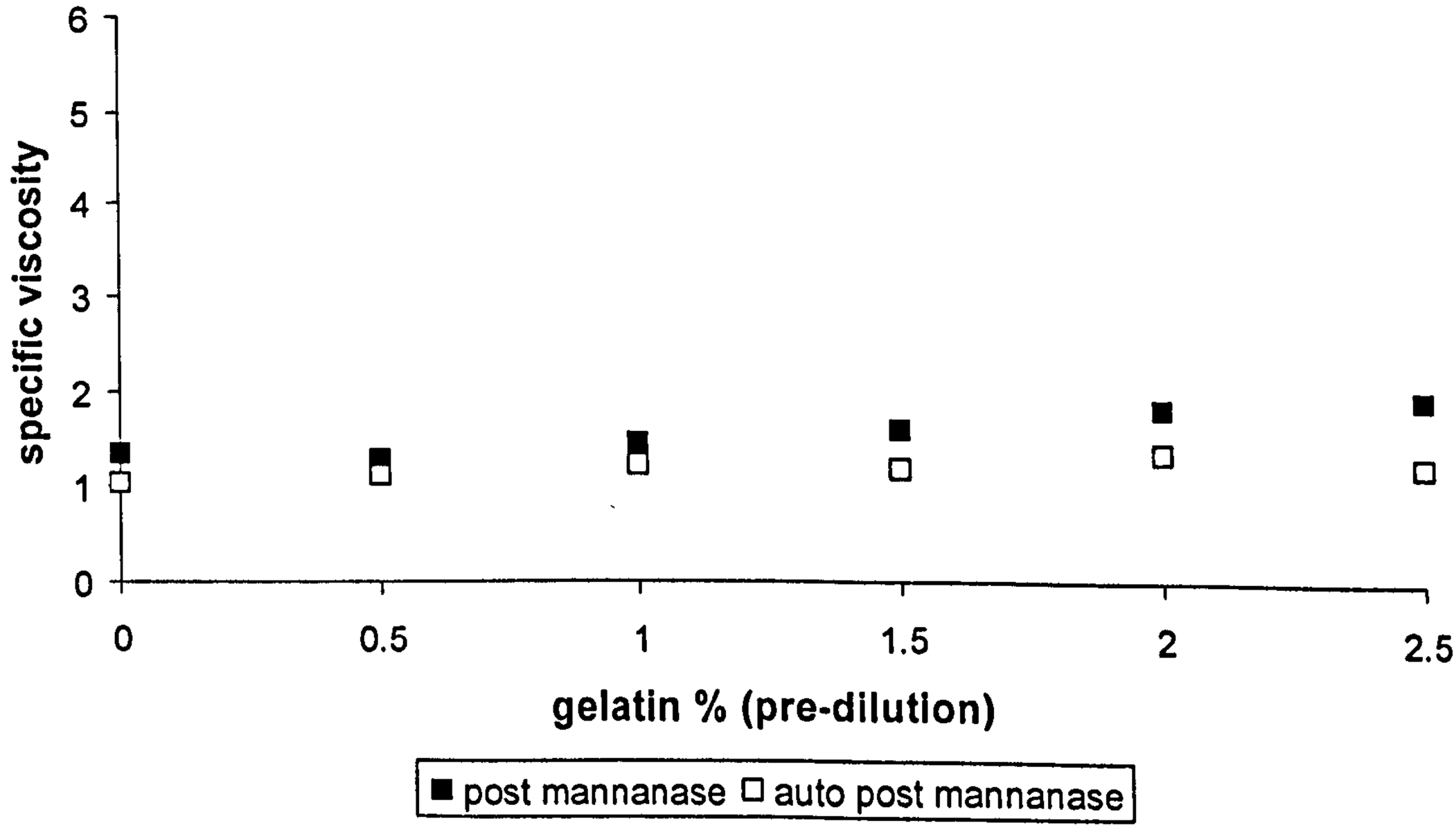


Figure 5-10 The effect of limed ossein gelatin on the viscosity of mannanase treated carrageenan/ locust bean gum mixtures before and after autoclaving.

Table 5-1 shows specific viscosity data for gelatin concentrations at 0 and 2.5% and across all heating and enzyme conditions for the data presented in figures 5-6 and 5-7. It is possible to gauge a very approximate contribution of each biopolymer component. Autoclaving the 0% gelatin sample causes an increase in the viscosity contribution of the post enzyme carrageenan from 22 to 35%. At 2.5% gelatin the contribution of the gelatin falls from 11 to 5% after autoclaving with the carrageenan component at a constant 17% of the total biopolymer viscosity. The reduction in carrageenan-specific viscosity can be explained partly by an increase in total viscosity, but the data shows a slight reduction in the presence of gelatin. The presence of gelatin results in an increase in proportion of viscosity attributable to LBG from 65 to 79%, supporting the view that LBG viscosity is maintained after autoclaving. The percentage viscosity contributions are given in table 5-2.

	Specific Viscosity			
	0% gelatin		2.5% gelatin	
Enzyme treatment	Non-autoclaved	Autoclaved	Non-autoclaved	Autoclaved
None	5.87	2.94	6.70	5.69
Mannanase	1.35	1.03	1.91	1.21
Mannanase and protease	1.29	1.04	1.17	0.92

Table 5-1 Specific viscosity values of non-autoclaved/autoclaved, enzyme treated, 0.3% carrageenan and 0.3% LBG mixtures at 0 and 2.5% limed ossein gelatin. Viscosity measured at 6 s^{-1} and at 50°C using double gap geometry after 1:1 dilution in sample buffer.

	% viscosity contribution			
	0% gelatin		2.5% gelatin	
	Non-autoclaved	Autoclaved	Non-autoclaved	Autoclaved
LBG	78	65	72	79
Gelatin	0	0	11	5
Carrageenan	22	35	17	16

Table 5-2 Percentage viscosity contributions for each biopolymer from the data in table 5-1.

5.3.4. Comparing the protective effect of other gelatins and proteins

The effect of the addition of 2.5% gelatin, sodium caseinate and glycine on the viscosity is shown in Table 5-3. The control viscosity after autoclaving represents 47% of the non-autoclaved value. Of the other samples glycine had a negligible effect at 53% of the non-autoclaved value. The greatest protective effect was observed for Collagel A. This was an enzyme hydrolysate of collagen and so had a very low molecular weight average as evidenced by its sol state at room temperature. This result shows that final viscosity receives no contribution from the protein component. A less pronounced effect was observed with Croda limed ossein at 69% the non-autoclaved value. The protein Na-caseinate had a limited protective effect at 59% of the non-autoclaved value. It is believed that the more compact structure of Na-caseinate may make it less liable to thermal degradation or to engage in antioxidant protection of the polysaccharides.

Having established that LBG was the most susceptible to a reduction in viscosity after autoclaving and that gelatin protected against this viscosity loss, the effect of different gelatins on LBG solutions were investigated. The removal of the carrageenan allowed the concentration of LBG to be increased. In Table 5-4 the effect of different gelatins at 2.5% on 0.8% LBG viscosity is shown. Fish gelatin had the greatest protective effect after autoclaving. This had an average viscosity of 70% of that of the non-autoclaved gelatin free control. This compares to 17% for the autoclaved control. Limed hide had the next highest post-autoclave viscosities at 64% of the control. A sample of 2.5% dried soluble meat extract was added. This was shown to be predominantly gelatin by hydroxyproline determination. This sample had some protective effect, giving post-autoclave viscosities above those for Sigma type b gelatin.

A comparison of the viscosity of the mixtures with that of gelatin alone shows no correlation and suggests that gelatin type, rather than molecular weight, is important. The values of isoelectric point reflect the chemical changes undergone during the processing of the gelatin. Native collagen has an isoelectric point of pH 9. This remains high unless reactions take place, which change the ratio of positive to

negative charges, such as deamidation (Mohammed, S.E. et al. 2000). Where lime $Ca(OH)_2$ was used in the process the IPs approach 5. In the case of the meat extract, the IP reduction is attributable to the reaction of the positively charged side groups of amino acids during the autoclave cycle.

Non-galactomannan components of LBG are believed to provide a level of protection during autoclaving. In order to estimate the effect of LBG purity on the final solution viscosity, a comparison of different LBG's was undertaken. Table 5-5 gives viscosity values at $1s^{-1}$ for 4 different LBG samples before and after autoclaving 60 minutes for 121°C and in the presence of 2.5% limed ossein gelatin. The highest pre-autoclaved viscosities, over 700 mPa.s, were recorded for the alcohol-precipitated samples, which reflects the higher proportion of galactomannan within the samples. The lowest pre-autoclaved viscosities were recorded for the industrial grade sample, which again relates to actual LBG concentration. After autoclaving, the reduction in viscosity was greatest for the Sigma sample at 9% the pre-autoclaved value, the lowest reduction being in the highly purified sample at 20% the pre-autoclaved value. The effect of gelatin was to increase the post-autoclave viscosity in all the samples. The greatest increase relative to the gelatin free post-autoclave value was with the industrial grade LBG, which had a viscosity that was 42% of the pre-autoclaved value. Gelatin had the lowest effect on post autoclave viscosity in the Sigma sample at 19% of the pre-autoclaved value, whilst the alcohol precipitate of the industrial grade LBG had a greater proportional reduction in viscosity, after autoclaving, compared to the non-precipitated industrial grade. The non-alcohol-precipitated Sigma sample had the greatest proportional reduction in viscosity. These results show that by increasing the purity of the starting gum, its ability to withstand autoclaving diminishes. This is consistent with recent literature (Kok, Hill et al. 1999). The viscosity reduction in the presence of gelatin indicates that the presence of non-galactomannan impurities still has an enhancing effect.

There are two hypotheses that explain the protective mechanism of gelatin in the experimental mixtures. Firstly, the reduction of degradation is due to an antioxidant property of the gelatin related to specific amino acid sequences. Alternatively, the

gelatin acts as a sacrificial agent, where degradation occurs, favouring its degradation rather than the polysaccharide.

The antioxidant property of gelatin has been discussed in the literature in relation to prevention of lipid oxidation. Enzyme hydrolysates of bovine skin and alaska pollack skin have been found to have a potent antioxidative activity on peroxidation of linoleic acid (Kim, Kim et al. 2001a), (Kim, Kim et al. 2001b). The antioxidative property of the hydrolysates was found to be amino acid sequence specific. Degradation in heated biopolymer solutions has been attributed to oxidative-reductive depolymerisation (Wellington 1983), which is of interest in the oil industry where biopolymers are used to suspend waste particles during drilling operations. It has been shown that the oxygen scavenger sodium sulphite at 1% maintains viscosity after autoclaving in 0.8% guar solutions (Paterson, Hill et al. 1997). Mixtures of oxygen scavengers and radical terminators can be used to stop/reduce molecular weight changes when autoclaving LBG (Mitchell and Hill 1995).

	Specific Viscosity	
	Non-autoclaved	Autoclaved
Control	5.43 (±0.06)	2.53 (±0.13)
Limed ossein	7.20 (±0.04)	4.99 (±0.14)
Collagel A	6.42 (±0.19)	5.30 (±0.35)
Na-caseinate	5.93 (±0.13)	3.51 (±0.07)
Glycine	4.49 (±0.35)	2.37 (±0.02)

Table 5-3 Comparison of autoclaved and non-autoclaved specific viscosity values of pre-digested 0.3% carrageenan and 0.3% LBG mixtures with 2.5% protein/amino acid. Viscosity measured at 6 s^{-1} and at 50°C using double gap geometry after 1:1 dilution in sample buffer. (Error ± 0.5*range of two replicates).

	Gelatin/LBG mixture viscosity * (mPa.s)	Gelatin viscosity (mPa.s)	Gelatin bloom value** (g)	Isoelectric point
LBG alone (not autoclaved)	207 (±4)	-	-	-
LBG alone	36 (±4)	-	-	-
Fish	147 (±4)	2.81	64	8.8
Limed hide	132 (±3)	6.41	285	5.0
Collagel A	107 (±5)	2.05	Non-gelling	7.6
Limed ossein	107 (±17)	6.15	305	5.0
Meat extract	92.3 (±14)	-	-	5.19
Sigma type b	82.2 (±4)	6.61	225	5.15

Table 5-4 Apparent viscosity values 0.8% LBG solution autoclaved with 2.5% different gelatins at $1s^{-1}$. Also shows viscosity of 6.66% gelatin solution at 40°C, bloom value of 6.66% gelatin gel at 10°C and isoelectric point value of 0.1% gelatin solution at 20°C. * A CV of 2.11% from three samples of non-autoclaved 0.8% LBG, prepared and measured on different days, shows a good reproducibility of the method. ** Manufacturers value

	Viscosity (mPa.s)		
	non-autoclaved	autoclaved	autoclaved + gelatin
Sigma	584 (±1)	51.5 (±4.5)	112 (±4)
Highly purified	714 (±3)	143 (±0)	248 (±37)
Industrial grade	285 (±7)	47.5 (±0.5)	107 (±17)
Alcohol ppt of industrial grade	765 (±5)	88.5 (±3.5)	247(±5)

Table 5-5 Viscosities at $1s^{-1}$ and 40°C of 0.8% w/w solutions of different LBG's before and after autoclaving at 60 minutes for 121°C and in the presence of 2.5% limed ossein gelatin. (Error ± 0.5*range of two replicates).

5.3.5. Determination of changes to LBG molecular using SEC-MALLS

The relationship between molecular weight and rheological behaviour allows us to make assumptions about what was happening to an autoclaved mixture of biopolymers on the basis of viscosity and gel analysis. In order to validate those assumptions, direct measurement of molecular weight was necessary. Size exclusion chromatography, with multi angle laser light scattering (SEC-MALLS), was selected for analysis of all biopolymers measured.

In terms of the experimental mixture used here, carrageenan was removed from the mixture for two reasons: firstly, for practical reasons as interpretation of SEC-MALLS spectra would have been made very difficult with a three-component system. Secondly, we had already been able to note that the changes to carrageenan were less significant than those of the LBG from the enzyme viscosity data.

5.3.5.1. Study with purified cLBG and limed ossein gelatin

Figures 5-11 and 5-12 show typical chromatograms obtained from the autoclaved samples. Initial concentration was 1% LBG and 2.5% Croda 305 gelatin, with the actual loaded concentrations being 3.5 and 1.0mg/ml respectively. There exists an inverse relationship between the required concentration for measurement and the molecular size of the sample. Insoluble material was removed after autoclaving by centrifugation. Figure 5-11 shows spectra of a mixture of gelatin and LBG autoclaved for 0, 5 and 60 minutes. Before autoclaving there is a light scattering peak at 14ml elution volume, with an average molecular weight of $1.253*10^6$Da, which can be attributed to LBG on the basis of its greater molecular weight. A second area combines a peak situated at 19ml, with a less distinct peak at 18ml. This bimodality is consistent with that expected for gelatin, which has a non-normal distribution based on the distinct polypeptide structures present. On the basis of peak height, the data shows that there is a higher proportion of lower molecular weight species. The average molecular weight for this bimodal region was $1.075*10^5$Da. After 5 minutes autoclaving time at 121°C the first peak was still present at 14ml, the molecular

weight being $9.989*10^6$Da. There was a change in the shape and position of the second peak. The bimodality was replaced by a more normal distribution and the peak volume was recorded at 19.5ml, suggesting a lowering of molecular weight. This was confirmed by the average of $6.176*10^4$Da. After 60 minutes the first peak had a volume of 14ml and a molecular weight average of $8.768*10^5$Da, whereas the second peak had a volume of just over 20ml and an average of $3.832*10^4$Da.

Figure 5-12 shows spectra of LBG alone autoclaved for 0, 5 and 60 minutes. A volume of 14ml was recorded for peaks at all autoclave times. The molecular weight average for the three samples were $1.029*10^6$Da, $7.428*10^5$Da and $7.690*10^5$Da.

In figure 5-13 average molecular weights for the full series of samples analysed using the SEC-MALLS technique are shown. This reveals that for the LBG peak of the mixture there is arguably no change in molecular weight with heating time, except perhaps between the first measurement interval, with values close to $1.0*10^6$Da. This is somewhat higher than has been previously observed for technical grade LBG by Kok 1999. The concentration data suggests an increase over time with values ranging from 0.35 to 0.53mg/ml at 0 and 60 minutes. This increase is believed to be associated with the post autoclave centrifugation of insoluble debris, which would have allowed this matter to further leach out LBG during the autoclaving. This LBG would have been of a higher molecular weight progressively and be increasingly less soluble. These concentrations can be converted to % recovery on the basis they represent a 10-fold dilution from the original starting %. In (b), the gelatin peak, there is the expected reduction in molecular weight. After 60 minutes this is less than 50% of the starting value of $9.0*10^4$ (±2.5)Da, there is a 30% reduction after 1 minute. The concentration data shows a constant trend around 2.4mg/ml across the heating times. The LBG only data in (c) had molecular weights that appear to be fairly stable across the heating times, even though these values were lower than those recorded for the mixture. The values were constantly around $8.3*10^5$Da, which is much closer to the value of $8.7*10^5$Da reported by Kok, 1999. There is less evidence for a concentration increase with stable values around 0.5mg/ml.

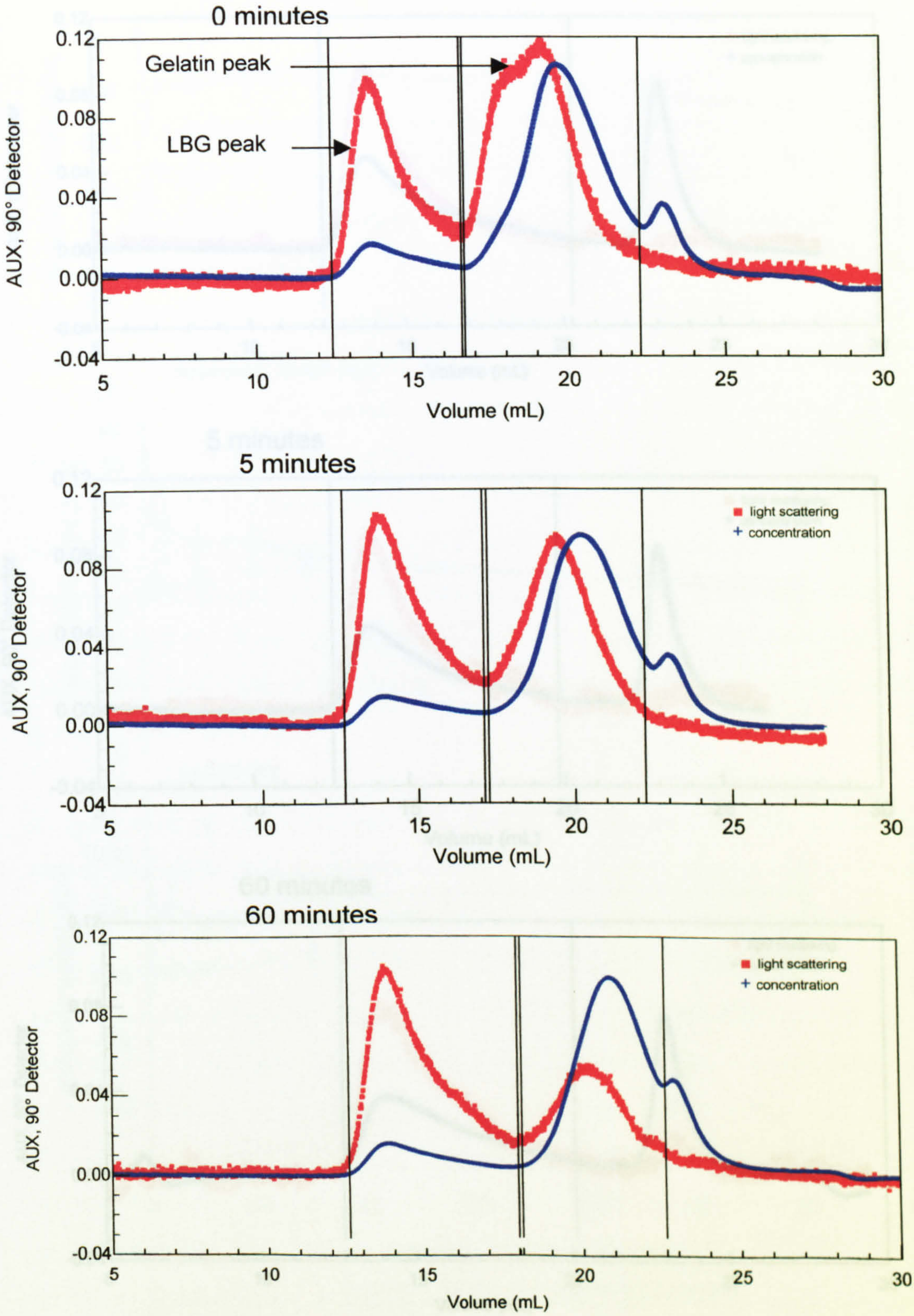


Figure 5-11 Light scattering (red) and refractive index (blue) elution patterns for gelatin/LBG mixtures autoclaved for 0, 5 and 60 minutes.

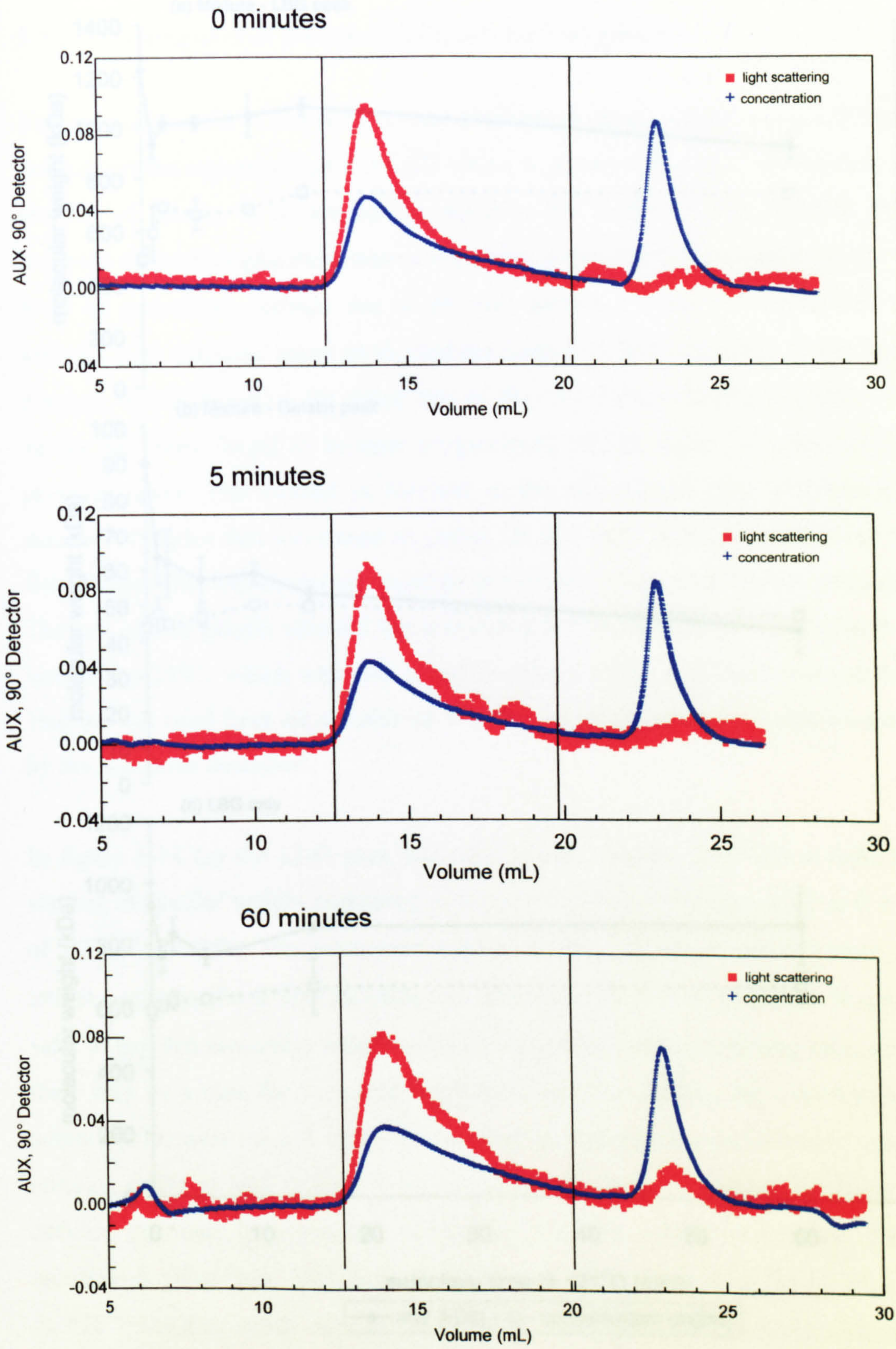


Figure 5-12 Light scattering (red) and refractive index (blue) elution spectra for LBG solution alone autoclaved for 0, 5 and 60 minutes.

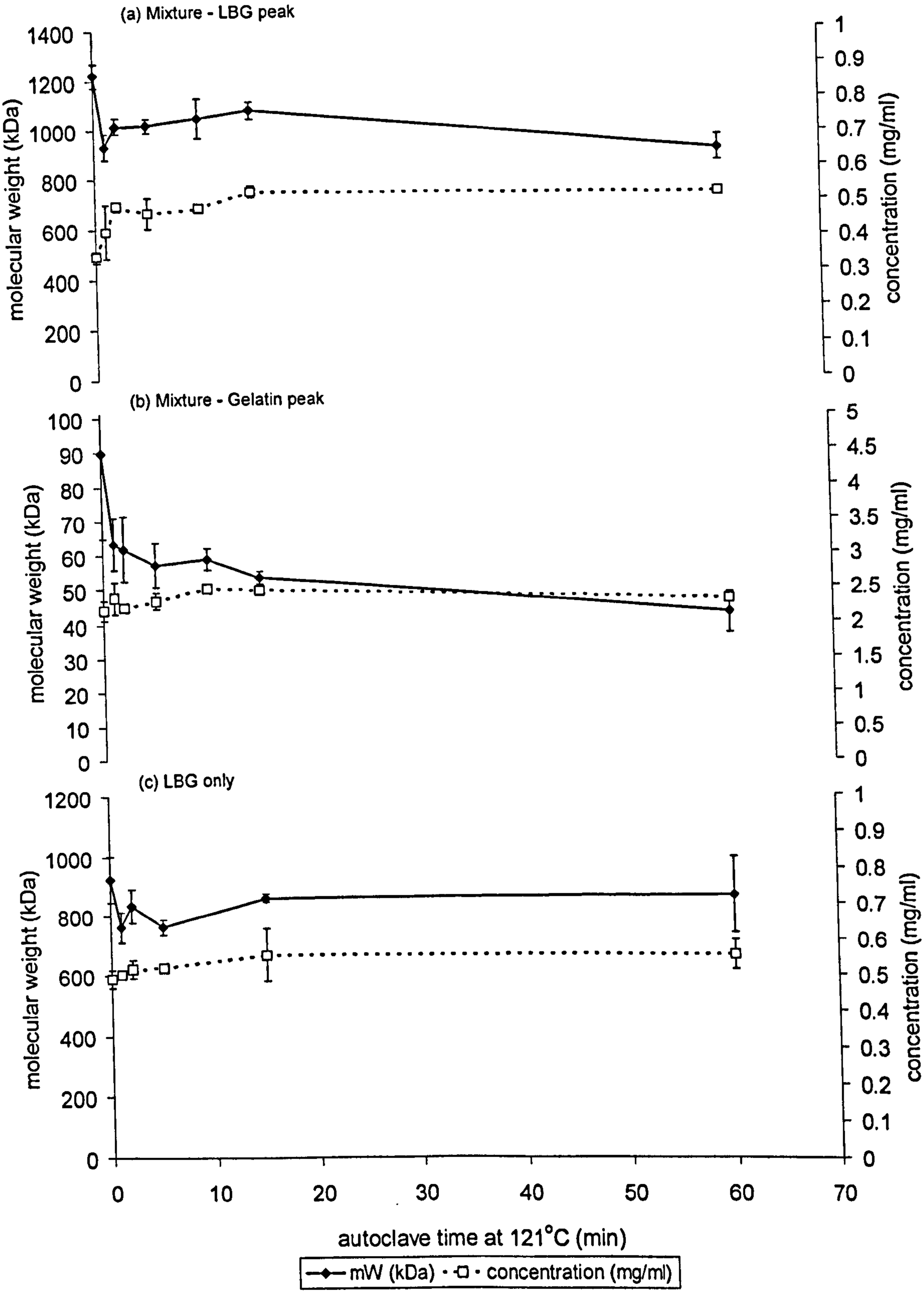


Figure 5-13 Average molecular weight and concentration values for 2.5% Croda limed ossein gelatin and 1% LBG samples autoclaved for differing times at 121°C in pH 6.8 phosphate buffer. Error shown SD of 3 replicates.

5.3.5.2. Using alcohol precipitate of cLBG and fish gelatin

The previous results in this section were obtained using essentially crude LBG and do not support the argument that it is LBG which is greatly affected by autoclaving in the absence of gelatin. In an attempt to reconcile this matter, it was believed that the presence of the non-galactomannan component of the cLBG was able to confer some level of protection, perhaps due to its own protein content. To verify this point, samples were prepared using an alcohol precipitate of the crude LBG. There was also a sound technical reason for doing this in that the light scattering detector baseline values had been found to become progressively higher during the duration of the previous study. This caused an increase in the noise signal ratio and lowered the number of angles that were used to gather the raw data. Also, it was decided to use fish gelatin. This had shown enhanced protective properties from the viscosity studies. The use of this gelatin allowed the measuring temperature of the SEC system to be lowered to 25°C, which was also found to reduce noise. A sample concentration of 1mg/ml was used from all samples on the basis of improvements to system sensitivity by the measures described.

In figure 5-14 (a) the LBG peak from the mixture shows there was a reduction in starting molecular weight compared to figure 5-13 with the mean value at 0 minutes of $5.7*10^5$ (±0.2)Da. The subsequent autoclave times suggest an upward trend from a low at 1 minute of $4.6*10^5$ (±0.2)Da, to a final value of $5.5*10^5$ (±0.2)Da. It is perhaps safer to say that molecular weight remains unchanged over autoclaving time, although there may be a case for increased solubility over time causing this observation. Any solubility increase would cause an increase in measured concentration; since this remains stable around 0.7mg/ml we may discount this explanation. In (b) we see a definite decrease in molecular weight for the fish gelatin over time. The pre-autoclaved value was 35% that of the limed ossein gelatin in figure 5-13 (b) at $3.2*10^4$ (±0.6)Da, which decreased to $1.9*10^4$ (±0.4)Da after 60 minutes. Figure 5-14 (c) shows that the LBG alone has a lower starting molecular weight than when in combination with gelatin, the value being $3.6*10^5$ (±0.3)Da. The only possible explanation for this is that aggregation may occur in the presence of gelatin, whereas the gelatin free mixtures allow greater freedom of LBG molecules to separate.

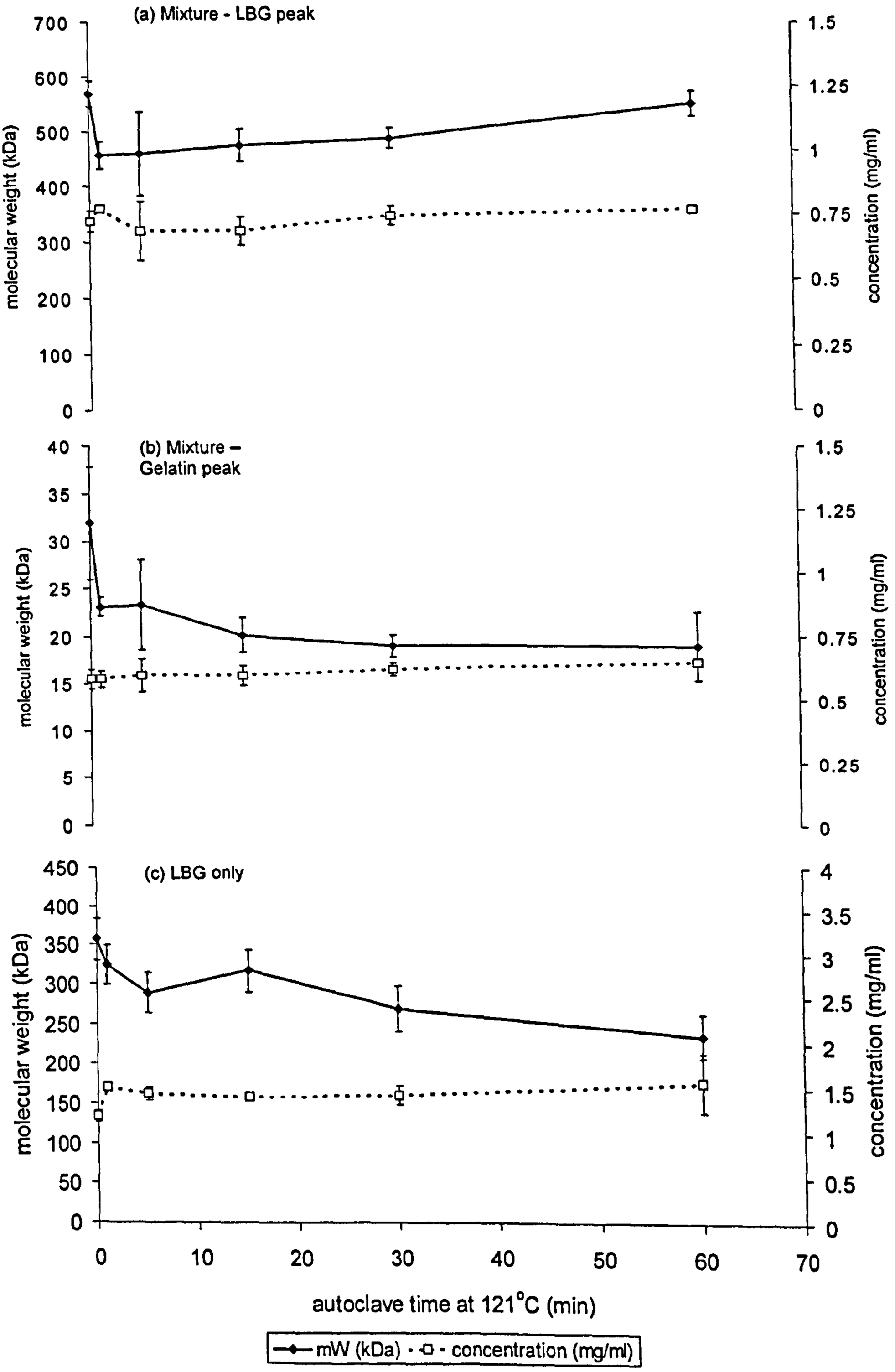


Figure 5-14 Average molecular weight and concentration values for 2.5% Croda fish gelatin and 0.8% alcohol ppt LBG samples autoclaved for differing times at 121°C in pH 6.8 phosphate buffer. Error shown SD of 3 replicates.

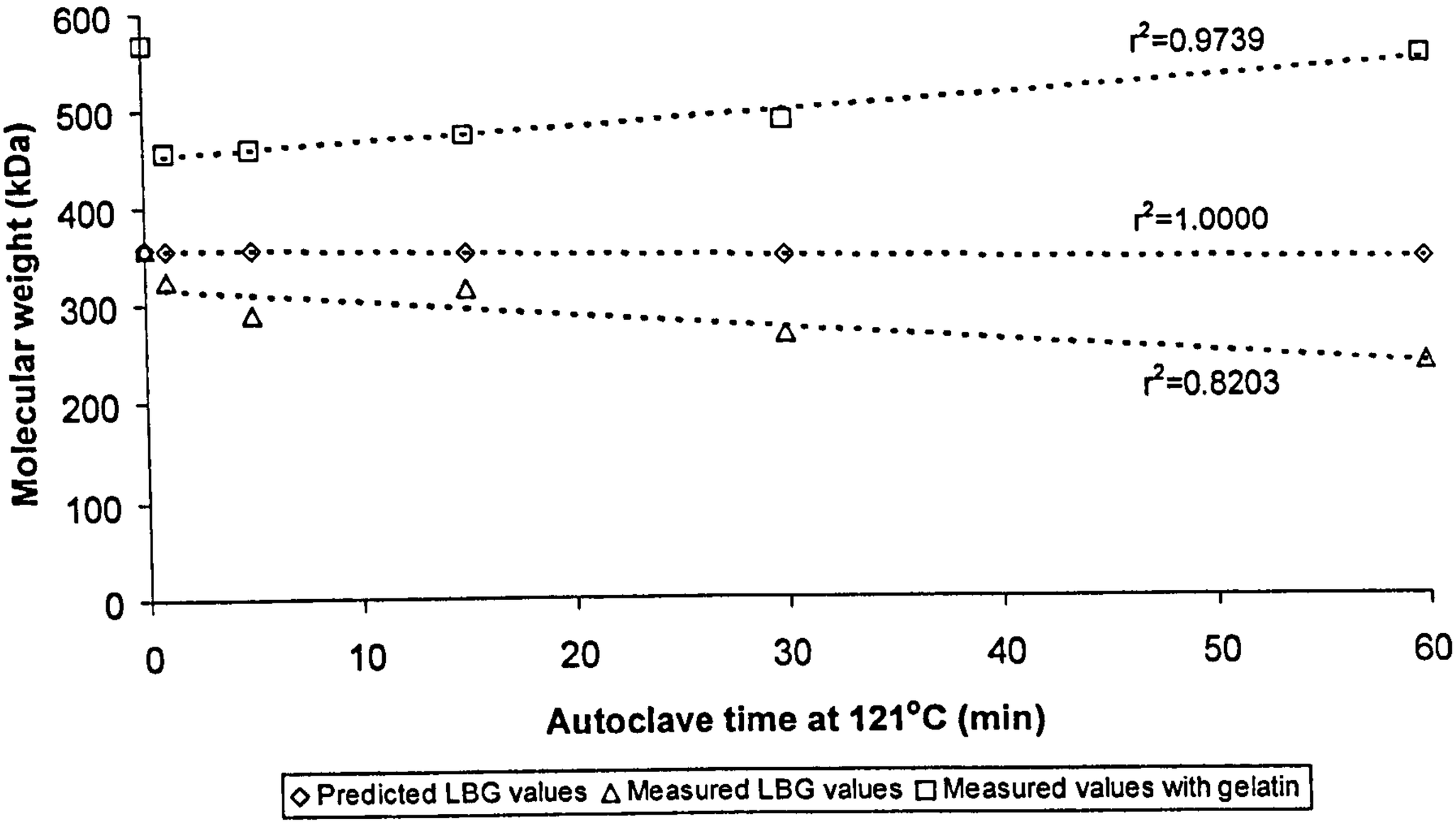


Figure 5-15 Measured molecular weight changes for LBG alone and the peak from the gelatin containing mixture. Also predicted changes for LBG using Kok's rate constant.

Lastly, a comparison was made of the data obtained with data predicted from a model of the reduction in molecular weight during a thermal process by the expression of Masson 1955:

$$\frac{1}{M_t} = \frac{1}{M_0} + \frac{k_D t}{m} \qquad \text{Equation 5-5}$$

Here M_0 and M_t are the molecular weights at time zero and t respectively, where t is the time, m is the weight of the monomer unit and k_D is the rate constant for degradation, the larger the rate constant the greater the extent of degradation. The predicted data was obtained using an activation energy at 121°C of 110kJ/mol, (Kok, Hill et al. 1999) and assuming the same starting molecular weight as the measured samples. The LBG used to derive the rate constant was an analytical grade obtained from Sigma. There are two interesting differences between the measured and predicted data for LBG alone: (1) the initial drop in molecular weight in the measured samples compared to the perfect linear fit to the origin for the predicted data, and (2) the higher residual value of the predicted data. In answering (1) the presence of super aggregated LBG clusters, which require pressure to break them up, would cause the sudden drop in molecular weight and indeed viscosity. Work conducted by Picout, Ross-Murphy et al. 2002 has shown the need for pressure assistance in accurately

measuring LBG molecular weight to achieve complete solubilisation. In response to (2) our sample was more highly purified due to the alcohol precipitation and would therefore have lost its internal protection mechanism. We would therefore expect a higher rate constant of degradation than for the predicted data.

Figure 5-15 shows experimental data for the alcohol precipitate of the centrifuged cLBG alone and in the presence of the fish gelatin. As with the viscosity data, linearity is apparent for all the autoclaved samples. The predicted data shows a greater resistance to autoclaving than the measured values, having a residual molecular weight of 95% compared to 65% for the measured data. There is evidence of an apparent increase in molecular weight from the mixture of gelatin and LBG. This is possibly due to an increase in measurable sample as autoclave time increases and is evidenced by the concentration data shown in figure 5-14a.

5.4. Conclusion

This chapter started with the hypothesis that the incorporation of gelatin enhanced the performance of the LBG/carrageenan system after autoclaving. This was tested using the buffer systems employed in petfood manufacture. Strong evidence was produced to show that gelatin did indeed produce a large protective effect after autoclaving, whereas, it weakened the gel in the non-autoclaved system. This latter observation suggested that the mechanisms for the improvement on gelatin incorporation were not due to phase separation or to the additional contribution of the gelatin gel to the overall gel strength. Rather, it suggested that gelatin incorporation protected the polysaccharides from degradation. To investigate this hypothesis, viscosity changes on autoclaving were measured in a "conventional" pH 6.8 phosphate buffer. To determine which biopolymer was responsible for the viscosity change, experiments were carried out with enzymes which degrade gelatin and locust bean gum. On the basis of these experiments it was concluded that locust bean gum was protected by the gelatin. To confirm that this was the case, experiments were carried out with SEC-MALLS. This showed that only for highly purified LBG was there degradation on autoclaving and protection by gelatin. For the system on which the viscosity work was carried out there was no evidence for LBG degradation on autoclaving, although gelatin clearly degraded. This raised the question of whether there was in fact a gel strength enhancement on gelatin addition in the pH 6.8 buffer in which the viscosity work and the SEC-MALLS was performed. Upon checking this it was found that in the pH 6.8 buffer gel strength loss on autoclaving and gelatin protection was much less pronounced than in the petfood TKPP buffer. An obvious experiment to do would be to repeat the SEC-MALLS work for this buffer system, but time constraints prevented this.

6. Final Discussion and Conclusions

The work in this thesis shows the dependence of gel texture on composition and thermal treatment of biopolymers commonly found in wet pet-foods. Studies on hydroxyproline content of the gel phase of retorted pet-food showed that up to 3% gelatin was present as a result of the leaching of collagen from the meat component. The significance of this level of gelatin was twofold; firstly, where samples were not subjected to retort conditions, gelatin had a negative effect on gel texture and secondly in retorted samples the presence of gelatin reduced the extent of gel weakening due to thermal degradation.

Gel weakening in non-retorted samples was originally believed to be a consequence of phase separation within the biopolymer mixture. Initially it was postulated that gelatin inclusions in a carrageenan/LBG continuous matrix might have acted as fracture initiation sites, promoting gel fracture. This was based largely on known incompatibility between LBG and gelatin. Evidence from gel curing studies showed that over time the gel strength increased, particularly at higher gelatin concentrations. This was believed to be due to the gelation of the gelatin inclusions, which then acted as re-enforcing filler. In real products, the gelatin concentration would be insufficient to provide any filler strength. At the upper end of the gelatin concentration encountered in product, its presence would lead to undesirable product performance. At the lower end it was believed that the mixture had a level of compatibility giving acceptable quality gels.

Further investigations into the biopolymer mixture revealed that the initial hypothesis might have not correctly described the underlying mechanism causing gel-weakening in non-autoclaved gels. Whilst incompatibility between LBG and gelatin was known and observed through viscometry, in mixtures containing carrageenan the question was as follows: assuming that carrageenan remains the dominant continuous biopolymer due to counter-ion entropy, which biopolymer was excluded? Evidence for a continuous carrageenan matrix was provided by small-deformation temperature studies. The assumption that LBG and carrageenan would both be found in the continuous phase was based on evidence from synergism between the two

biopolymers upon gelation. The hypothesis that LBG does not show a specific interaction with the carrageenan matrix rather than specific binding might help explain why it is possible for LBG to be excluded (Cairns, Miles et al. 1987). The driving force for the exclusion of LBG is twofold; firstly, the known incompatibility between LBG and gelatin (Alves, Garnier et al. 2001) and secondly, the partial compatibility between carrageenan and gelatin. In fact, phase separation in carrageenan-gelatin systems is usually associative with the formation of ionic complexes. This association is dependent on system pH and the IEPpH of the gelatin (Michon, Cuvelier et al. 1995).

The most likely structure possible is a continuous matrix of carrageenan and gelatin with excluded domains of LBG. The nature of phase separation is that the higher molecular weight fractions are excluded first. Indeed the greater compatibility between carrageenan-gelatin may be in part due to the lower molecular weight of the gelatin compared to the LBG. So then, high molecular weight LBG being excluded, this will diminish the positive effect of LBG on the carrageenan matrix, regardless of the actual mechanism responsible. We are now in a state where the polysaccharide concentration required for gelation is reduced, and since this was always at the low end for practical and cost reasons, gel weakening occurs. All is not lost, however, if we are aware that gelatin can itself form a synergistic gel with carrageenan. Indeed work has been conducted in fish gelatin-carrageenan mixtures to increase the melting temperature (Haug, Williams et al. 2003) of the gel. It would appear that this is less important than the weakening due to loss of LBG from the carrageenan domain.

The work conducted, using samples that been subjected to autoclaving, showed a positive effect on the gel strength of adding gelatin to the biopolymer mixture investigated in this thesis. One hypothesis was that the gelatin possessed some special property by which it protected the polysaccharides from degradation. An assumption was made that since gel strength is related to molecular weight and that thermal degradation reduces molecular weight of biopolymers, the gel strength reduction was caused by a reduction in the molecular weight of the gelling biopolymers. It was also thought that since oxidative-reductive depolymerisation was the principal mechanism for molecular weight reduction in autoclaved biopolymer solutions, then some property must exist to reduce or affect this. One idea to explain why gel strength was

higher in gelatin-containing mixtures than 'cook-outs' using just the polysaccharides was that gelatin acted as a sacrificial agent based on its greater susceptibility to degradation. If there was a finite amount of bond breakage in a heated system then gelatin would break rather than would LBG/carrageenan. This seemed plausible given the poor performance of gelatin gels after heating. An alternative hypothesis was that gelatin possessed some antioxidant property and actively scavenged degrading free radicals. The evidence for this was derived from studies using skin enzyme hydrolysates in preventing lipid oxidation, (Kim, Kim et al. 2001). Whilst some of the work indicated that LBG viscosity was protected to different extents depending on gelatin type, there was no evidence to suggest such a mechanism was in existence. The area of antioxidant properties of gelatin in retorted aqueous biopolymer systems is an area of interest, and further work would be beneficial.

Whilst the idea of gel strength reduction being due to molecular weight loss was attractive, the evidence from SEC-MALLS did not bear this out. This was probably because the assumption that the LBG in these systems was fully solubilised was not justified. The concept of super-aggregates of galactomannans that are difficult to solubilise is one that has received some attention recently (Picout, Ross-Murphy et al. 2002),(Picout, Ross-Murphy et al. 2002). These aggregates would have molecular weights of many million Daltons and would be unlikely to be measured using light scattering due to their removal by the guard column. Their effect on viscosity would be considerable, and perhaps intrinsic viscosity measurements would have been a useful addition to the data presented.

Two questions were raised regarding the results gained from this work:

Is viscosity in fact an indication of molecular weight, or is related to something else? If the latter were true then the conclusions in the earlier part of chapter 5 would be invalid.

Why was there a difference in the behaviour of the mixtures produced in the two buffer systems?

These two questions bring us to the heart of the work and are the focus of this part of the discussion.

In answer to question 1, as shown in Chapter 1, there is a well-recognised relation between [η] and molecular weight. For many polysaccharides the viscosity of more concentrated solutions can be generalised by a single plot of log c [η] versus log $\eta_{specific,}$ (Morris, Cutler et al. 1981). Locust bean gum does not fit this general relationship, giving higher viscosities than expected because of "hyper-entanglements". Essentially LBG shows an extensive tendency to aggregate. This is shown not only by the higher than expected viscosities, but also by the very values of the Huggins constant (Gaisford, E. et al. 1986) and the data reported by Richardson, Clark et al. 1999. Picout and co-workers have shown that LBG and other galactomannans do not produce true solutions unless subjected to autoclaving temperatures (Picout, Ross-Murphy et al. 2002). This raises the possibility that the viscosity changes observed could be related not to the primary molecular weight, but to changes in the state of aggregation. If, however, this hypothesis is to be preserved it is necessary to explain why:

a) It does not occur in the TKPP buffer.

b) The addition of other biopolymers, such as gelatin, appears to reduce the LBG viscosity loss.

The latter is probably not too difficult to justify since the addition of a second biopolymer would be expected to promote association of the first biopolymer on thermodynamic criteria. It is possible, therefore, that viscosity changes could be interpreted in ways other than primary molecular weight reduction.

An alternative explanation is that the SEC-MALLS data is made on a sample which is not representative of the viscosity data. There could be two possibilities here:

a) The total sample used for the SEC-MALLS experiments were not representative of the samples used for the viscosity measurements.

b) The guard column took out highly aggregated material, which did show molecular weight changes, but this component never reached the main column.

Recovered or measured concentration of LBG was ~70%, therefore up to 30% LBG was unaccounted for. It is plausible that this 30% consisted of the super-aggregated LBG.

What is the difference between the two buffers?

If the hypothesis is retained that gel strength reduction is due to LBG degradation, then the gel strength data suggested that this is far more pronounced in the high ionic strength buffer. We suggested that there could be two reasons for an ionic strength dependence on degradation. Firstly, the SEC-MALLS data for the pure LBG compared with the cruder material suggest that "impurities" have a protective effect on the galactomannans. This has been shown from other work as well. It is not unreasonable to hypothesise that the distribution of these protective ingredients could be ionic strength dependent. Basic considerations using the DLVO theory would suggest that at high ionic strength such material, if electrically charged, would flocculate in the presence of higher levels of salt. The ionic strength was 0.5M for TKPP compared to 0.1M for pH 6.8 phosphate buffer. Such flocculated material would be less available to act as a scavenger of oxygen and/or free radicals and therefore have a less protective effect. Thus in the TKPP buffer the behaviour, in terms of degradation, is closer to the pure material. This is of course a hypothesis that could be tested experimentally through further work. The second reason for an ionic strength dependence on degradation can be explained as follows. Kok has postulated that aggregated LBG is less susceptible to degradation than the soluble biopolymer, (Kok 1999). It might be expected that high ionic strength would maintain the aggregates due to salting out. However, it is well recognised, (Piculell, Iliopoulos et al. 1991), that where there is a mixture of a non-polyelectrolyte, such as LBG, and a charged material, then a high ionic strength produces a homogeneous phase as opposed to phase separation. This is because of counter-ion entropy effects. In such a homogeneous solution LBG will be less aggregated than in a phase separated system.

If this is indeed the story behind the findings here then the presence of LBG aggregates is responsible for gel strength enhancement, and their protection by soluble proteins is essential in this system.

In terms of aiding our understanding of the exact causes of gel weakening in these mixtures, further work should be conducted. One possibility is to use microscopy to identify the phases present and to measure the size of the domains under changing

conditions. This would be the most reliable indicator of phase behaviour, as any attempt to separate the phases before gelation had occurred would not give a representative reflection. Following on from this, an important piece of further work would be to investigate the phase composition of autoclaved gelled mixtures. This would reveal the importance of changes to LBG during heating and whether reducing the molecular weight of gelatin during the autoclaving cycle would promote compatibility, as is believed. This would explain why LBG integrity, whether as free coils or super-aggregates, was important in gel texture.

The industrial significance of the findings of this work are concerned with how gel texture is produced and how it may vary either in a negative or positive. In less severe thermal conditions, phase separation of the constituent biopolymers may be reduced by the omission of LBG and the creation of a gelatin enhanced carrageenan gel. The texture of the final gel should be more compliant than a pure carrageenan gel. Fairly recent work in fish gelatin/carrageenan mixtures shows the possibility of producing desirable textures between carrageenan and low molecular weight gelatin, (Haug, Delve et al. 2001).

This concludes the final discussion and indeed this thesis.

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